AD_____

Award Number: W81XWH-€Ì ÊF ĚeÏ G G

TITLE: Þ^¸ ÁÒ}:ˆ{ ^ÁÚ¦[å¦ˇ *Áæ å ÁT ^c@ä } ä ^ËÖ^]|^cä }ÁÔ[{ àä æ æ ä }Á/@¦æ ^Á¦¸ÁÓ¦^æ •cÁÔæ }&^¦ÄÖ^•ã }^åÁ¦¦ÄÒ~^&ã ^ÄÖ^|ã ^¦ˆÁ Á@Á˘{ [¦

PRINCIPAL INVESTIGATOR: Ü[*^¦Á Pæ ¦ã [}

CONTRACTING ORGANIZATION: University of U\|æ @{ æ
          Þ[¦{ æ }ÉÁUSÁ Î H€FJÁ

REPORT DATE: U&q à^¦Á G€FF

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
          Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
01-10-2011	Annual	15 SEP 2010 - 14 SEP 2011

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
New Enzyme Prodrug and Methionine-Depletion Combination Therapy of Breast Cancer Designed for Effective Delivery to the Tumor	
	5b. GRANT NUMBER
	W81XWH-08-1-0722
	5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)	5d. PROJECT NUMBER
Roger Harrison	
	5e. TASK NUMBER
E-Mail: rharrison@ou.edu	5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
University of Oklahoma Norman, OK 73019	

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Recombinant L-methioninase-annexin V and cytosine deaminase-annexin V fusion proteins have been produced in good purity and yield from E. coli, with both FP genes of the correct sequence. As indicated by measuring the dissociation constant (Kd), both purified FPs bind strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown in vitro. In vitro tests of both enzyme prodrug systems showed that significant killing of endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells (~80% confluent) was obtained with very little or no effect of the prodrug when the FP was not present. The L-methioninase-annexin V/SeMet enzyme prodrug system was tested in vivo in nude mice with implanted MDA-MB-231/GFP cancer cells using i.p. injection of the FP and the prodrug. The result was that the tumors were nearly completely eliminated. These results provide strong support for the idea that this enzyme prodrug system will lead to the elimination of breast tumors wherever they occur in the body.

15. SUBJECT TERMS
Enzyme prodrug and methionine-depletion therapy for breast cancer, tumor vasculature targeting, L-methioininase-annexin V/selenomethionine enzyme prodrug, cytosine deaminase/annexin V/5-fluorocytosine enzyme prodrug

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U	UU	31	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

The focus of this project is on the development of new ways to treat breast cancer with minimal or no side effects. There are two aims of the project: (1) Develop a novel enzyme prodrug and methionine-depletion combination cancer therapy in which the enzyme L-methioninase is targeted by the human protein annexin V to the breast tumor vasculature, using selenomethionine as the prodrug, and (2) develop a novel enzyme prodrug cancer therapy in which the enzyme cytosine deaminase is targeted by the human protein annexin V to the breast tumor vasculature, using 5-fluorocytosine as the prodrug. Annexin V is known to bind with high affinity to phosphatidylserine (PS) in phospholipids bilayers. PS has recently been shown to be expressed on the external surface of endothelial cells that line the blood vessels in tumors but is not expressed on the external surface of the vascular endothelium in normal organs. The enzyme L-methioninase catalyzes the conversion of methionine to methanethiol, α-ketobutyrate, and ammonia. It also catalyzes the conversion of selenomethionine (SeMet) to toxic methylselenol, α-ketobutyrate, and ammonia. Methylselenol has been shown to be cytotoxic to various cancer cells. Cytosine deaminase converts the prodrug 5-fluorocytosine to the cytotoxic drug 5-fluorouracil. To accomplish the specific aims of this project, the L-methioninase-annexin V and cytosine deaminase-annexin V fusion proteins (FPs) will be produced and purified, the strength of binding of each FP to human endothelial cells and two breast cancer cell lines *in vitro* will be determined, the cytotoxicity *in vitro* of each FP in combination with its respective prodrug will be determined for endothelial cells and two breast cancer cell lines, and the L-methioninase-annexin V/selenomethionine enzyme prodrug combination will be tested in nude mice with tumor xenografts (two breast cancer cell lines) for its effect as an enzyme prodrug by itself and also in combination with methionine-depletion therapy.

BODY

The research accomplishments for the third year of this project are summarized as follows:

Task 1 – Production of the recombinant L-methioninase-annexin V fusion protein to be tested

For use in tests with nude mice during year 3 of the project, the L-methioninase-annexin V FP with the correct gene sequence was produced as needed by recombinant expression in *E. coli* and purified using the same procedures described in our publication (attached) about this research [1], with the exception that a wash of the immobilized metal affinity chromatography column with the FP bound was performed with 0.1% Triton X-114 to remove endotoxin. The result was a reduction in endotoxin by greater than a factor of 10^6 and a low level of endotoxin in the purified protein (< 100 EU/mg).

Task 2 – Test of the function *in vitro* of annexin V in the L-methioninase-annexin V fusion protein

The strength of binding and stability of binding over 3 days of this FP for the three cell lines was reported in the annual report for year 2 and also in our publication about this research, which is attached [1]. The results for the dissociation constant K_d to measure the strength of binding indicate that the binding of the FP to these cells is relatively strong and that the binding to the endothelial cells is stronger than for either of the two breast cancer cell lines. The data for binding stability indicates that the binding of FP declined over 3 days for all three cell lines, with the MDA-MB-231 cancer cells showing the most rapid decline; however, the FP was still present at day 3 for all three cell lines.

Task 3 – Test of the anticancer activity *in vitro* of the L-methioninase-annexin V fusion protein in combination with selenomethionine prodrug on endothelial cells and breast tumor cells

Results of the ability of the enzyme prodrug system to eliminate human endothelial cells and breast cancer cells were given in the annual report for year 2 and also in our publication about this research, which is attached [1]. Significant killing of the cells (~80% confluent) was found at a SeMet concentration of 500 µM for endothelial cells, 50 µM for MCF-7 breast cancer cells, and 10 µM for MDA-MB-231 breast cancer cells. With no FP present, significant cell killing was not observed for the endothelial cells and MDA-MB-231 cancer cells at up to 1000 µM SeMet and for MCF-7 cancer cells at up to 500 µM SeMet.

Task 4 – Test of the anticancer activity *in vivo* of the L-methioninase-annexin V fusion protein in combination with selenomethionine prodrug

To date, we have performed four tests of this enzyme prodrug therapy in nude mice, which are described as follows:

Test 1 - This test was performed using MDA-MB-231 breast cancer cell line transfected with green fluorescent protein (GFP) that were obtained from Cell Biolabs, Inc. (San Diego, CA). The treatment groups used for this test were as follows (7-8 mice/group), with FP and SeMet each injected at 10 mg/kg and with 2-3 $\times 10^6$ cells injected per mouse:
  1. Control group - saline on days 0-9
  2. FP group - FP on days 0 and 5, saline on days 1-4 and 6-9
  3. SeMet group - saline on days 0 and 5, SeMet on days 1-4 and 6-9
  4. Treatment group - FP on days 0 and 5, SeMet on days 1-4 and 6-9
The cells were injected in the left flank with an equal volume of Matrigel. The result was that only two one tumors grew in group 1, and one tumor grew in each of groups 2, 3, and 4. We concluded that more cells needed to be injected for all of the tumors to grow.

Test 2 - MDA-MB-231/GFP cells were again used, with the number of cells injected increased to ~5 $\times 10^6$ with 6 mice per group, using the same treatment groups as in test 1. The time

between the injections of FP and SeMet was reduced from 24 hours in the first test to 14 hours in this test. This resulted in two mice dying in group 4 and one mouse dying in group 3, so the test was terminated half way through the first round of treatment. We concluded from this test that there should not be less than 24 hours between SeMet injections.

Test 3 - MDA-MB-231/GFP cells were again used, with the number of cells injected increased to ~8×10^6 with 5 mice per group, using the following groups, with FP and SeMet each injected at 10 mg/kg:
1. Control group - saline on days 0-11
2. FP group - FP on days 0, 4, and 8; saline on days 1-3, 5-7, and 9-11
3. SeMet group - saline on days 0, 4, and 8; SeMet on days 1-3, 5-7, and 9-11
4. Treatment group - FP on days 0, 4, and 8; SeMet on days 1-3, 5-7, and 9-11

The mean tumor volume as a function of days after inoculation of tumor cells is shown in Figure 1. While the tumors in the three control groups continued to grow slowly after treatment, the tumors in the FP + SeMet treatment group decreased to near zero, and at the end of the test, only one mouse in this group had a tumor. The mean tumor volume for the FP + SeMet group became significant compared to the control group at 41 days since inoculation, denoted by * ($p < 0.001$); a one-way ANOVA was used for statistical analysis using GraphPad InStat software (GraphPad; La Jolla, CA). The size of the tumor in the FP + SeMet group was 7% of the mean size of the tumors in the control group. Between days 32 and 49 after inoculation, one mouse died in the FP + SeMet group and two mice died in the SeMet group, which we believe is caused by SeMet toxicity. This borderline toxicity problem was discussed with Dr. Carla Kurkjian, Collaborator at the University of Oklahoma Health Sciences Center in the Hematology/Oncology Section, and we have concluded that for future tests the SeMet injections should be reduced to a dosage of 5 mg/kg. The slow growth of the tumors in the control groups was also a problem, and we concluded that this could be due to the high passage number of the MDA-MB-231/GFP tumor cells.
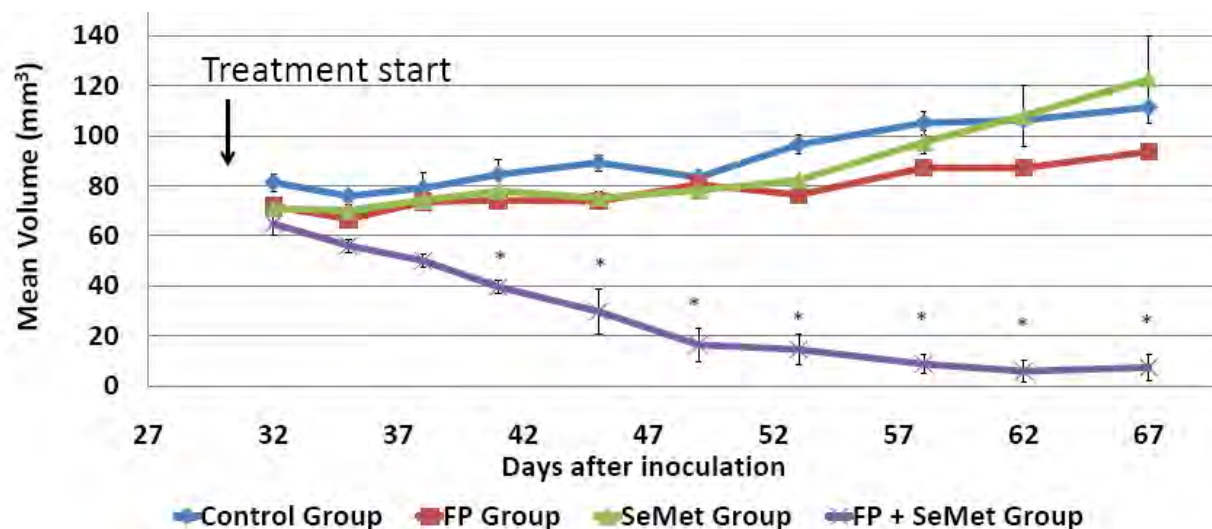
Figure 1. Results of enzyme prodrug treatment of nude mice with implanted MDA-MB-231/GFP tumors. The mean tumor volume for the FP + SeMet group became significant compared to the control group at 41 days since inoculation, denoted by * ($p < 0.001$).

Test 4: We used MDA-MB-231 cells grown from a new vial obtained from Cell Biolabs, and we used a strain transfected with red fluorescent protein (RFP), in order to visualize the cells well using a new small animal fluorescence imaging system where the animal tests are being performed. We used the same treatment groups and approximately the same number of cells as in test 3. After 30 days, no tumors appeared, so we terminated this test. Therefore, we have concluded it is highly likely that the burden of expressing either GFP or RFP is causing the MDA-MB-231 cells to grow significantly slower. We have seen papers in the literature where MDA-MB-231 cells not transfected with GFP or RFP grew to larger sizes in nude mice than shown in Figure 1 in a shorter length of time (220 mm^3 in 43 days in a paper by Zhang et al. [2]; 160 mm^3 in 24 days in a paper by Sadej et al. [3]). Therefore, we plan to test this enzyme prodrug treatment with untransfected MDA-MB-231 cells. At the end of treatment, any metastases to the liver and lungs will be confirmed by microscopic evaluation on formalin-fixed sections stained with H&E, and the number of metastatic nodules in the lungs will be determined after fixation using a dissecting microscope.

We have tested a procedure to quantify the exposure of phosphatidylserine on the surface of the tumor vasculature in nude mice with MDA-MB-231 tumors. Biotinylated FP was injected i.p., and after 2 hours, a peristaltic pump was used to perfuse the blood vessels with heparinized saline with 2 mM Ca^{2+}. There was a problem with cutting the vena cava during this procedure. We have consulted about this problem with Dr. Rajagopal Ramesh, Director of Experimental Therapeutics and Translational Cancer Medicine at the University of Oklahoma Health Sciences Center, and he suggested using a dissecting microscope that is available in the facility used to

house the nude mice, which we plan to do. He also suggested that we could use a syringe to push the saline through, instead of a pump.

Task 5 - Production of the recombinant cytosine deaminase-annexin V fusion protein

The results of the production and purification of the cytosine deaminase-annexin V FP are described in our publication about this research, which is attached [4]. The purity of the FP was estimated to be >96% using densitometry software analysis The theoretical molecular weight of the FP monomer is 53 kDa, which is consistent with the SDS-PAGE result for the purified FP. The lyophilized FP was determined to have a cytosine deaminase specific activity of 18 U/mg of protein, which is consistent with the specific activity of purified cytosine deaminase that has been reported in the literature. The overall recovery yield of cytosine deaminase activity was found to be >90%.

Task 6 - Test of the function *in vitro* of annexin V for the cytosine deaminase-annexin V fusion protein

The strength of binding and stability of binding over 3 days of this FP for the three cell lines are reported in our publication about this research, which is attached [4]. The dissociation constant (K_d) for measuring the strength of binding for each cell line grown to ~80% confluence tested was obtained from the specific binding data to give the following results: 1.5 ± 0.2 nM, endothelial cells, 0.6 ± 0.4 nM for MCF-7 breast cancer cells, and 4.2 ± 1.8 nM for MDA-MB-231 breast cancer cells, indicating relatively strong binding between the FP and PS. In a control test, the specific binding assay was performed on endothelial cells that were 100% confluent, and the result was that the total and non-specific data were essentially identical, indicating no specific binding and therefore a lack of PS expression on the cell surface. The data for binding stability shows that the FP per cell declined over 3 days; however, binding was still measureable after 3 days.

Binding was also studied using the FP labeled with FITC (fluoroscein isothiocyanate) [4]. A fluorescence image of MDA-MB-231 cells shows that the FP is bound to the surface of the cells and not internalized. By remaining on the cell surface and not being internalized, where it would be digested, the FP is able to convert 5-FC to 5-FU and thus lead to cytotoxicity of the cell and other cells surrounding it, by a bystander effect. Endothelial cells incubated with FITC-labeled FP were also observed to be fluorescent only at the cell surface when they were 70% confluent. No fluorescence was observed when endothelial cells were 100% confluent, indicating no binding of the FP and thus no expression of PS on the surface of the cells.

Task 7 - Test of the anticancer activity *in vitro* of the cytosine deaminase-annexin V fusion protein in combination with 5-fluorocytosine prodrug on endothelial cells and the two breast tumor cell lines

Results of the ability of the cytosine deaminase-annexin V/5-fluorocytosine enzyme prodrug system to eliminate human endothelial cells and breast cancer cells (~80% confluent) are

presented in our publication about this research, which is attached [4]. For the endothelial cells, significant killing was observed on day 9 for cells with FP plus 5-FC (p < 0.001), and killing was observed to be dose-dependent.   The two breast cancer cell lines exhibited similar patterns of cell death. Both MCF-7 and MDA-MB-231 cells receiving the FP plus 5-FC showed significant cell killing that was dose-dependent, compared to the control group, on days 3, 6, and 9 (p < 0.001). By day 9, treatment with the FP plus 5-FC resulted in 4% and 12% cell viability at 1000 µM 5-FC for MCF-7 and MDA-MB-231 cancer cells, respectively. For all three cell lines, no significant cell death was found with 5-FC present and FP absent, or with 5-FC absent but FP present.

KEY RESEARCH ACCOMPLISHMENTS

- Purified recombinant L-methioninase-annexin V fusion protein (FP) was produced in good purity (>97%) and yield (80 mg/liter of starting culture broth). Two mutations in the FP gene were corrected using site-directed mutagenesis.
- As indicated by measuring the dissociation constant (K_d), purified L-methioninase-annexin V binds strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown *in vitro*. Hydrogen peroxide was found to not be needed to induce exposure of PS on non-confluent endothelial cells. The following K_d values were determined: 0.5 nM for endothelial cells, 6.2 nM for MCF-7 cells, and 4.9 nM for MDA-MB-231 cells. The amount of L-methioninase-annexin V bound on the three cell lines *in vitro* was found to decline steadily over 3 days, but there was still some FP bound at day 3.
- In tests of the L-methioninase-annexin V/SeMet enzyme prodrug system *in vitro*, significant killing of the cells (~80% confluent) was found at a SeMet concentration of 500 µM for endothelial cells, 50 µM for MCF-7 breast cancer cells, and 10 µM for MDA-MB-231 breast cancer cells. With no FP present, significant cell killing was not observed for the endothelial cells and MDA-MB-231 cancer cells at up to 1000 µM SeMet and for MCF-7 cancer cells at up to 500 µM SeMet.
- A time profile of L-methioninase-annexin V in the bloodstream after i.p. injection in nude mice showed that the FP diminished to an undetectable level 8 hours after injection. The peak level of the FP was approximately 10 times greater for an injection of 10 mg/kg than for 1 mg/kg.
- In a test of the effectiveness of the L-methioninase-annexin V/SeMet enzyme prodrug system *in vivo* in nude mice, implanted MDA-MB-231/GFP cancer cells were almost completely eliminated using i.p. injection of the FP and the prodrug. While the tumors in the three control groups continued to grow slowly after treatment, the tumors in the FP + SeMet treatment group decreased to near zero, and at the end of the test, only one mouse in this group had a tumor. The size of the tumor in the FP + SeMet group was 7% of the mean size of the tumors in the control group (no treatment).
- Purified recombinant cytosine deaminase V fusion protein was produced in good purity (>96%), yield (50 mg/liter of starting culture broth), and specific activity (18 U/mg of protein). DNA sequencing verified that the gene sequence is correct.
- Purified cytosine deaminase V binds strongly to human endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells grown *in vitro* to ~80% confluence,

as indicated by measuring the dissociation constant (K_d),. The following K_d values were determined: 1.5 nM for endothelial cells, 0.6 nM for MCF-7 cells, and 4.2 nM for MDA-MB-231 cells. Using FP with FITC attached, binding to the surface of MDA-MB-211 cells was observed by fluorescence microscopy. Endothelial cells incubated with FITC-labeled FP were also observed to be fluorescent only at the cell surface when they were 70% confluent; no fluorescence was observed when endothelial cells were 100% confluent, indicating no binding of the FP and thus no expression of PS on the surface of the cells. This result for binding to endothelial cells indicates that dividing cells mimic exposure of PS in the tumor vasculature, while confluent cells mimic PS exposure in the normal vasculature. The binding of cytosine deaminase-annexin V to the three cell lines *in vitro* was found to decline steadily over 3 days, but there was still some FP bound at day 3.

- In tests of the cytosine deaminase-annexin V/5-FC enzyme prodrug system *in vitro* with ~80% confluent cells, significant killing of endothelial cells was observed on day 9 for cells with FP plus 5-FC. The two breast cancer cell lines exhibited similar patterns of cell death. Both MCF-7 and MDA-MB-231 cells receiving the FP plus 5-FC showed significant cell killing that was dose-dependent, compared to the control group, on days 3, 6, and 9 ($p < 0.001$). By day 9, treatment with the FP plus 5-FC resulted in 4% and 12% cell viability at 1000 µM 5-FC for MCF-7 and MDA-MB-231 cancer cells, respectively. For all three cell lines, no significant cell death was found with 5-FC present and FP absent, or with 5-FC absent but FP present.

REPORTABLE OUTCOMES

Brent D. Van Rite, Yahya A. Lazrak, Magali L. Pagnon, Naveen R. Palwai, Peter S. McFetridge, and Roger G. Harrison, Enzyme Prodrug Therapy Designed to Target L-Methioninase to the Tumor Vasculature, Cancer Letters, 2011, 301, 177-184.

Brent D. Van Rite and Roger G. Harrison, Annexin V-targeted Enzyme Prodrug Therapy Using Cytosine Deaminase in Combination with 5-Fluorocytosine, 2011, Cancer Letters, 307, 53-61.

Brent D. Van Rite, Mohamad Cherry, Carla Kurkjian, Vassilios Sikavitsas, and Roger G. Harrison, "Targeted Enzyme Prodrug Therapy to Treat Breast Cancer," Biomedical Engineering Society Annual Meeting, Hartford, CT, October, 2011.

Van Rite, B.D., Lazrak, Y.A., Pagnon, M., McFetridge, P.S., Sikavitsas, V.I., Bose, P., Kurkjian, C., and Harrison, R.G. (speaker), "Novel Enzyme Prodrug Therapy of Breast Cancer Designed for Effective Delivery to the Tumor," Era of Hope 2011 Breast Cancer Research Conference, Orlando, FL, August, 2011.

Van Rite, B.D., Lazrak, Y.A., Pagnon, M., McFetridge, P.S., Sikavitsas, V.I., Bose, P., Kurkjian, C., and Harrison, R.G. (speaker), "Novel Enzyme/Prodrug for Targeting and Treatment of Solid Breast Tumors," American Institute of Chemical Engineers Annual Meeting, Salt Lake City, November, 2010.

Brent D. Van Rite, Yahya A. Lazrak, Magali Pagnon, Vassilios I. Sikavitsas, Prithvi Bose, Carla Kurkjian, and Roger G. Harrison, "A Novel Enzyme/Prodrug for Targeting and Treatment of Solid Breast Tumors," Biomedical Engineering Society Annual Meeting, Austin, TX, October, 2010.

Brent D. Van Rite (speaker), Magali Pagnon, Yahya Lazrak, and Roger G. Harrison, "The Use of Targeted Enzyme/Prodrugs for the Treatment of Solid Tumors," 39th Annual Biochemical Engineering Symposium, Kansas State University, Manhattan, KS, April, 2010.

Harrison, R.G., "Enzyme Prodrug Cancer Therapy Selectively Targeted to Tumor Cells or Tumor Vasculature and Methods of Production Thereof," U.S. Patent Application, March, 10, 2010.

Yahya A. Lazrak, Brent D. Van Rite, Magali Pagnon, Arafat Tfayli, Peter S. McFetridge, and Roger G. Harrison (speaker), "Targeting the Vasculature of Solid Tumors with Enzyme/Prodrug and Methionine-Depletion Therapy," American Institute of Chemical Engineers Annual Meeting, Nashville, November, 2009.

Harrison, R.G., Van Rite, B.D., Lazrak, Y.A., Pagnon, M., and McFetridge, P.S., "Targeting of Solid Tumor Vasculature with Enzyme/Prodrug and Methionine-Depletion Therapy," Biomedical Engineering Society Annual Meeting, Pittsburgh, October, 2009.

Harrison, R.G., "Enzyme Prodrug Cancer Therapy Selectively Targeted to Tumor Cells or Tumor Vasculature and Methods of Production Thereof," U.S. Provisional Patent Application, March, 2009.

Lazrak, Y.A. (speaker), Neves, L.F.F., McFetridge, P.S., and Harrison, R.G., "Novel Enzyme-Prodrug Therapy for Cancer," oral presentation, Biomedical Engineering Society Annual Meeting, St. Louis, October, 2008.

Harrison, R.G. (speaker), Lazrak, Y.A., Neves, L.F.F., McFetridge, P.S., and Tfayli, A., "Novel Enzyme-Prodrug Therapy for Cancer," American Institute of Chemical Engineers Annual Meeting, Philadelphia, November, 2008.

CONCLUSION

Recombinant L-methioninase-annexin V and cytosine deaminase-annexin V fusion proteins have been produced in good purity and yield from *E. coli*, with both FP genes of the correct sequence. Both FP's have been shown to bind strongly to PS exposed on human endothelial cells and MCF-7 and MDA-MB-231 breast cancer cells (dissociation constants ranging from 0.5 to 6.2 nM). Using FITC-labeled cytosine deaminase-annexin V, binding on the surface of MDA-MB-231 breast cancer cells was observed by fluorescence microscopy. Human endothelial cells incubated with FITC-labeled FP were also observed to be fluorescent only at the cell surface when they were 70% confluent; no fluorescence was observed when endothelial cells were 100% confluent, indicating no binding of the FP and thus no expression of PS on the surface of the cells. This result for binding to endothelial cells indicates that dividing cells

mimic exposure of PS in the tumor vasculature, while confluent cells mimic PS exposure in the normal vasculature. In a binding experiment over 3 days for each of the three cell lines, it was found that there was a steady decline in both FP's bound over this period, but there was still some FP bound at day 3. *In vitro* tests of both enzyme prodrug systems showed that significant killing of endothelial cells, MCF-7 breast cancer cells, and MDA-MB-231 breast cancer cells (~80% confluent) was obtained with very little or no effect of the prodrug when the FP was not present.

      The L-methioninase-annexin V/SeMet enzyme prodrug system was tested *in vivo* in nude mice with implanted MDA-MB-231/GFP cancer cells using i.p. injection of the FP and the prodrug.   The result was that the tumors were nearly completely eliminated by the enzyme prodrug treatment. While the tumors in the three control groups continued to grow slowly after treatment, the tumors in the FP + SeMet treatment group decreased to near zero, and at the end of the test, only one mouse in the this group had a tumor. The size of the tumor in the FP + SeMet group was 7% of the mean size of the tumors in the control group (no treatment). In order to have faster growing control cancer cells in future *in vivo* tests, we plan to test this enzyme prodrug treatment with untransfected cancer cells.

      The *in vitro* and *in vivo* results to date provide strong support for one of the basic ideas for the project, which is that the conversion of the SeMet to methylselenol at the surface of the tumor vasculature will lead to damage of the tumor vasculature; this damage will lead to clotting of the tumor vasculature, thus cutting off the oxygen supply of the cancer cells. In addition, the breast cancer cells will be killed by toxic methylselenol being carried across the artery wall by fluid permeation. The effect of methylselenol on normal cells outside of the tumor is expected to be minimal or none because it will be greatly diluted by the bloodstream before it reaches the normal cells. Also, SeMet in the bloodstream will not be converted to methylselenol in the normal vasculature, because PS will not be exposed, and the SeMet will be held at a level that will not damage the normal vasculature. Therefore, side effects should be minimal.

REFERENCES

1. Van Rite, B.D., Lazrak, Y.A., Pagnon, M.L., Palwai, N.R., Neves, L.F., McFetridge, P.S., and Harrison, R.G., Enzyme Prodrug Therapy Designed to Target L-Methioninase to the Tumor Vasculature, 2011, Cancer Letters, 301, 177-184.

2. Zhang, H. et al., Dual Activity Lysophosphatidic Acid Receptor Pan-antagonist/Autotaxin Inhibitor Reduces Breast Cancer Migration *In vitro* and Causes Tumor Regression *In vivo*, 2009, Cancer Res., 69, 5441-5449.

3. Sadej, R. et al., 2009, CD151 Regulates Tumorigenesis by Modulating the Communication between Tumor Cells and Endothelium, Mol. Cancer Res., 7, 787-798.

4. Van Rite, B.D. and Harrison, R.G., Annexin V-targeted Enzyme Prodrug therapy Using Cytosine Deaminase in Combination with 5-Fluorocytosine, 2011, Cancer Letters, 307, 53-61.

APPENDIX I

Brent D. Van Rite, Yahya A. Lazrak, Magali L. Pagnon, Naveen R. Palwai, Peter S. McFetridge, and Roger G. Harrison, Enzyme Prodrug Therapy Designed to Target L-Methioninase to the Tumor Vasculature, 2011, Cancer Letters, 301, 177-184.

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

CANCER
LETTERS

Enzyme prodrug therapy designed to target L-methioninase to the tumor vasculature

Brent D. Van Rite, Yahya A. Lazrak, Magali L. Pagnon, Naveen R. Palwai, Luís F.F. Neves, Peter S. McFetridge, Roger G. Harrison *

Bioengineering Center and the School of Chemical, Biological and Materials Engineering, University of Oklahoma, Norman, OK, USA

ARTICLE INFO

ABSTRACT

A new approach for enzyme prodrug therapy for cancer was tested using human endothelial cells and two breast cancer cell lines in vitro. The concept is to use the human annexin V protein to selectively target the enzyme L-methioninase to the tumor vasculature. The major finding was that enzyme prodrug treatment using the L-methioninase-annexin V fusion protein and selenomethionine as the prodrug over 3 days was shown to be lethal to the endothelial cells and the cancer cells, while having little or no effect with the prodrug but with no fusion protein present. Thus, this new approach appears promising.

© 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Enzyme prodrug therapy for cancer was conceived as a way to avoid the systemic toxicity of chemotherapy. A type of enzyme prodrug therapy known as antibody-directed prodrug therapy (ADEPT) was first proposed in the 1980s as a means to confine the action of cytotoxic drugs to the tumor. In ADEPT, a tumor-associated antibody is linked to a drug-activating enzyme, and the resulting fusion protein is administered systemically and preferentially accumulates in the tumor [1]. A non-toxic prodrug is administered systemically and is converted in the tumor to a toxic drug by the enzyme. The enzyme should not have a human homolog to avoid prodrug activation in normal tissues. Although this system is appealing in principle, there have been clinical limitations of ADEPT, including

poor accessibility of the enzyme/antibody complex to the tumor [2].

To improve on the ADEPT concept for cancer treatment, we have developed a new approach that targets the enzyme prodrug therapy to the tumor vasculature. In this approach, human annexin V protein is fused to the enzyme moiety and used to bind to the anionic phospholipid phosphatidylserine (PS) on the surface of endothelial cells in the tumor vasculature. PS is typically exposed on the surface of the vasculature endothelium within blood vessels of tumors, but not on normal endothelium [3,4]. Also, it has been shown that annexin V, when injected systemically, localizes in the tumor vasculature and tumor cells [3,4]. For the enzyme component, we have chosen L-methionine gamma-lyase (accession #AAB03240), also known as L-methioninase, from *Pseudomonas putida*. This enzyme catalyzes the α,γ-elimination of L-methionine and selenomethionine and is not found in human tissue [5]. Methionine is converted to methanethiol, α-ketobutyrate, and ammonia, while selenomethionine is cleaved into toxic methylselenol, α-ketobutyrate, and ammonia [6]. Methylselenol has been shown to be approximately 200-fold more

* Corresponding author. Address: School of Chemical, Biological and Materials Engineering, University of Oklahoma, 100 E. Boyd, Room T335, Norman, OK 73019, USA. Tel.: +1 405 325 4367.
E-mail address: rharrison@ou.edu (R.G. Harrison).

cytotoxic to various human cancer cells than its prodrug [7] and is known to induce apoptosis in cancer cells [8,9].

Several mechanisms of action of this new system are envisioned. The methylselenol generated at the surface of the endothelial cells in the tumor leads to destruction of these cells, which leads to clotting in the tumor vasculature and a cutoff of the supply of oxygen to the tumor. Thus, the primary tumor and distant metastases can be treated simultaneously. Methylselenol is also carried to the tumor cells by fluid permeating through the artery wall because of the pressure gradient across the artery wall. The destruction of the endothelial cells releases tumor antigens directly in the bloodstream, which can cause the immune system to mount a systemic attack on any remaining tumor cells anywhere in the body; this immune response can be boosted by the administration of immunoadjuvants [10]. Finally, the ʟ-methioninase will greatly reduce the supply of methionine in the tumor, which will weaken methionine-dependent cancer cells.

Using the methioninase-annexin V fusion protein as an enzyme prodrug system, here we report the characterization of this system by *in vitro* studies using human endothelial cells and two breast cancer cell lines that includes a determination of the dissociation constant (K_d) of binding to the cell surface, the stability of the binding, and an evaluation of the cytotoxicity of the enzyme prodrug system.

2. Materials and methods

2.1. Materials

Oligonucleotide primers were synthetically produced by the Molecular Biology Resource Facility at the Health Sciences Center of the University of Oklahoma. Linear pET-30 Ek/LIC vector, T4 DNA polymerase, HRV 3C protease, and NovaBlue and BL21(DE3) *E. coli* cells were obtained from Novagen (Madison, WI). BamHI restriction enzyme and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). HAAE-1 endothelial cells were from Coriell Cell Repositories (Camden, NJ). MCF-7 and MDA-MB-231 cells and cell culture media were obtained from the American Type Culture Collection (Manassas, VA). Streptavidin-HRP was purchased from KPL (Gaithersburg, MD). PCR and plasmid purification kits were from Qiagen (Vista, CA). Alamar Blue solution was obtained from BioSource (Camarillo, CA).

2.2. Construction of recombinant expression plasmid

The expression vector pET-30 Ek/LIC/METHANX, encoding the methioninase-annexin V fusion protein (FP), was constructed in the following manner: The DNA sequences encoding for the FP were amplified from pKK223-3/ATF-Meth [11] and pET-22b(+)/STFANX (obtained from Dr. Stuart Lind at the University of Colorado) by the polymerase chain reaction using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). The PCR primers used to create the fusion protein gene, connected by a flexible linker, were as follows:

(a) 5′ primer for ʟ-methioninase: 5′-*GAC/GAC/GAC/AAG/*ATG/**CTT/GAA/GTC/CTC/TTT/CAG/GGA/CCC**/CGC/GAC/TCC/CAT/AAC/AAC/ACC-3′ (b) 3′ primer for ʟ-methioninase: 5′-GC/CGC/ATT/<u>GGA/TCC</u>/AGA/ACC/GCT/GCC/TGC/ACA/CGC/CTC/CAA/CGC/CAA/CTC/G-3′ (c) 5′ primer for annexin V: 5′-CG/ATT/CGC/<u>GGA/TCC</u>/GCA/CAG/GTT/CTC/AGA/GGC-3′ (d) 3′ primer for annexin V: 5′-*GA/GGA/GAA/GCC/CGG/*TTA/GTC/ATC/TTC/TCC/ACA/GAG/C-3′. The ʟ-methioninase primers incorporated a 5′ LIC cloning site (italics), an HRV 3C protease site (bold), and a 3′ BamHI site (underlined). The annexin V primers added a 5′ BamHI site (underlined) and a 3′ LIC cloning site (italics).

The PCR products were purified using the QIAquick PCR purification kit, digested with BamHI restriction enzyme, and purified with the same kit. The pure, digested genes were ligated using T4 DNA ligase and run on an agarose gel. Using the QIAquick gel purification kit, the proper fragments were cut from the gel and purified. The pure methioninase-annexin V fusion gene was annealed to the pET-30 Ek/LIC linear vector using T4 DNA polymerase to create sticky ends and was transformed into competent NovaBlue cells. After successful transformation, plasmids containing the proper fusion gene insert were extracted from the NovaBlue cells using the QIAprep plasmid purification protocol and transformed into *E. coli* BL21(DE3) to be used as the host for protein expression. The final vector contains integrated thrombin and enterokinase cleavage sites, an N-terminal His-tag sequence for easy purification, and an engineered HRV 3C protease cleavage site that cleaves the sequence LEVLFQ↓GP at the start of the methioninase-annexin V gene. The sequence of the FP gene was verified to be correct by DNA sequencing at the Oklahoma Medical Research Foundation (Oklahoma City, OK).

The expression vector pET-30 Ek/LIC/ANX that encodes annexin V was constructed using the same methods as for the pET-30 Ek/LIC/METHANX expression vector, as follows: The DNA sequence encoding for annexin V was amplified from pET-22b(+)/STFANX by the polymerase chain reaction, using two primers: (a) 5′ primer: 5′-*GAC/GAC/GAC/AAG/*ATG/**CTT/GAA/GTC/CTC/TTT/CAG/GGA/CCC**/GCA/CAG/GTT/CTC/AGA/GGC-3′ and (b) 3′primer: 5′-*GA/GGA/GAA/GCC/CGG/*TTA/GTC/ATC/TTC/TCC/ACA/GAG/C-3′.

2.3. Expression and purification of recombinant protein

Recombinant methioninase-annexin V FP and annexin V were produced and purified using the procedure of Zang [11]. This procedure uses immobilized metal affinity chromatography (IMAC) with immobilized Ni^{2+} to isolate the FP or annexin V. After cleavage by HRV 3C protease, purified FP or annexin V is collected in the flow-through of another IMAC. Any uncleaved FP or annexin V and HRV 3C protease, which also contains a His-tag, remained bound to the IMAC column.

2.4. Protein content and enzymatic activity determination

The Bradford assay from Bio-Rad (Hercules, CA) was used for all protein determinations throughout the purification using bovine serum albumin (BSA) as the standard. Samples were analyzed by denaturing gel electrophoresis

using the SDS–PAGE method with Coomassie blue staining [12]. The L-methioninase enzyme activity was measured using L-methionine as a substrate by the spectrophotometric determination of α-ketobutyrate with 3-methyl-2-benzothiazolone hydrazone hydrochloride [5].

2.5. Cell culture

Human HAAE-1 aortic endothelial cells were grown in F-12K medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS), 0.03 mg/ml endothelial cell growth supplement, and 0.1 mg/ml heparin. MCF-7 human breast cancer cells were maintained as monolayer cultures in Eagle's minimum essential medium containing Earle's balanced salt solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and supplemented with 10% FBS and 0.01 mg/ml bovine insulin. MDA-MB-231 human breast cancer cells were grown in Leibovitz's L-15 medium supplemented with 10% FBS and 2 mM L-glutamine. Penicillin (100 U/ml) and streptomycin (100 μg/ml) were also added to each medium. HAAE-1 and MCF-7 cells were grown at 37 °C in a 5% CO_2 atmosphere, while MDA-MB-231 cells were grown without additional CO_2 at 37 °C, as recommended by the American Type Culture Collection.

2.6. Protein binding assay

Each cell line was grown to 70–80% confluence in T-75 flasks. Cells were transferred to 24-well culture plates (5×10^4 cells/well) and grown to 80–85% confluence. Cells were fixed to the plate using 0.25% glutaraldehyde in binding buffer (PBS with 2 mM Ca^{2+}). Excess aldehyde groups were quenched using 50 mM NH_4Cl in binding buffer. Varying concentrations of biotinylated FP or annexin V, using SureLINK Chromophoric Biotin (KPL) in a 60 M excess during biotinylation, were diluted in binding buffer containing 0.5% BSA and incubated at 37 °C for 2 h. After washing with binding buffer with 0.5% BSA, streptavidin-HRP was added at 2 μg/ml and incubated at room temperature for 1 h. Following washing with binding buffer, HRP was measured by adding the chromogenic substrate O-phenylenediamine (0.4 mg/ml) and hydrogen peroxide (0.012 vol.%) in 0.05 mM phosphate-citrate buffer (pH 5.0). After 30 min at room temperature in the dark, the solution was transferred to a transparent 96-well plate, and the absorbance was read at 450 nm on a BioTek Synergy HT microtiter plate reader (Winooski, VT). All experiments had a blank that was subjected to the same procedure but with no FP or annexin V added. To determine non-specific binding, the same procedure was performed with no Ca^{2+} and 5 mM EDTA in the binding buffer, with the addition of a 1 h BSA (0.5%) pretreatment for the cancer cells prior to adding the FP or annexin V.

2.7. Binding stability assay

To assess how long the FP remains bound to the surface of the endothelial cells, a modified binding assay was used. Cells on 24-well plates were first incubated for 2 h at 37 °C

in a saturating concentration of biotinylated FP (100 nM) in complete growth medium with 2 mM Ca^{2+}. The Alamar Blue assay (see *In vitro* enzyme prodrug cytotoxicity to cells) was done on separate sets of cells at days 0–3 to determine viability, followed by fixing with 0.25% glutaraldehyde in binding buffer. Excess aldehyde groups were quenched by incubation in a 50 mM NH_4Cl in binding buffer. The binding of FP was then quantified using streptavidin-HRP and OPD as above.

2.8. In vitro enzyme prodrug cytotoxicity to cells

The experiment was carried out over 3 days, using the same cells for each of the days. Cells were grown and plated in 24-well plates with respective growth media using the same procedure as for the FP binding assay (see above). Each medium was supplemented with 2 mM Ca^{2+} and 0.02 mM pyridoxal phosphate (since annexin V is Ca^{2+} dependent and pyridoxal phosphate is a cofactor for L-methioninase). On day 0, the cells were incubated in medium containing 100 nM FP for 2 h at 37 °C. The plates were washed and medium containing SeMet varying from 0 to 1000 μM was added. The Alamar Blue assay was performed on all wells on day 1. The Alamar Blue assay was performed by adding Alamar Blue solution to each well to give 10% Alamar Blue and then incubated for 4 h at 37 °C. The solution was transferred to an opaque 96-well plate, and the fluorescence was read at 590 nm using excitation at 530 nm. The blank consisted of wells containing only medium and Alamar Blue solution. After the fluorescence reading, the plates were washed, replaced with fresh medium containing appropriate levels of SeMet, and placed in the incubator. The readings were taken every 24 h for the duration of the experiment.

2.9. Data analysis

All assays included wells in triplicate. To test differences in cell viability, a one-way ANOVA employing a Tukey–Kramer multiple comparisons test was performed using GraphPad InStat software (GraphPad; La Jolla, CA) with a significance level of $p < 0.001$.

3. Results

3.1. Protein expression and purification

An SDS–PAGE gel of the FP purification is shown in Fig. 1. Following expression of the fusion protein, the bacteria cells were harvested by centrifugation, resuspended in lysis buffer and sonicated. The resulting soluble protein fraction is shown in lane 1. The His-tagged fusion protein was bound to the Ni^{2+} column during the first chromatography step to allow some of the unwanted proteins to flow through into waste (lane 2), and then it was eluted (lane 3). After cleavage with the protease, the FP eluted during the flow-through (lane 4) of the second chromatography, while other proteins remained in the column. Dialysis of the flow-through, sterile filtration, endotoxin removal, and lyophilization ended the purification process. The yield of purified FP was 30 mg from 1 l of culture medium. The purity of the FP (lane 4) was estimated to be >97% using UN-SCAN-IT densitometry software analysis (Silk Scientific, Inc.). The theoretical molecular weight of the FP monomer is 80 kDa, which is consistent with the SDS–PAGE result for the purified FP (lane 4). The lyophilized FP was determined to have a methioninase specific activity of 1.0 U/mg of
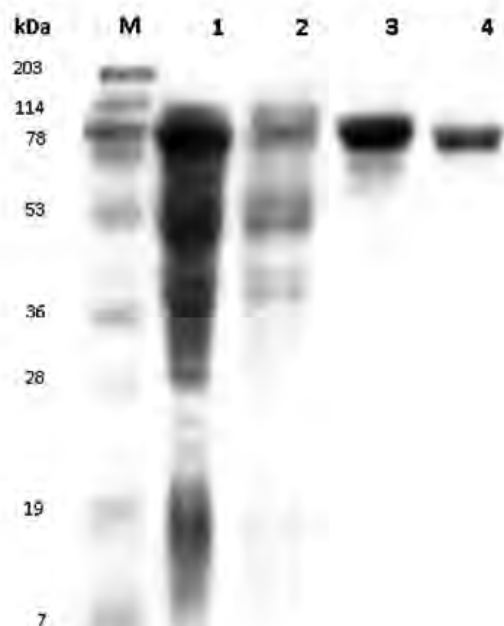
Fig. 1. SDS–PAGE gel of methioninase-annexin V purification. An 8% gel with Coomassie blue staining was used to determine approximate molecular mass of 10 μl of purified samples. Lane 1, soluble proteins; lane 2, first chromatography flow-through; lane 3, first chromatography elution; lane 4, second chromatography flow-through; M marker proteins with molecular masses indicated in kiloDaltons (5 μl).

protein. The overall recovery yield of L-methioninase activity was found to be 50%. L-methioninase activity remained relatively constant when stored in lyophilized form at −80 °C.

Annexin V was expressed and purified using the same procedures as for the FP. The yield of purified annexin V was 80 mg per liter of culture medium, and the protein purity was estimated to be >97% using UN-SCAN-IT densitometry software analysis. The theoretical molecular weight of annexin V (35 kDa) is consistent with an SDS–PAGE analysis for purified annexin V (data not shown).

3.2. Specific binding and dissociation constant determination

The ability of the FP to bind to endothelial cells and breast cancer cells with PS exposed on the cell surface was evaluated by equilibrium binding experiments in which increasing concentrations of biotinylated FP were used. In initial experiments with endothelial cells, hydrogen peroxide was used at a low concentration (1 mM) to induce exposure of PS. In later experiments, the H_2O_2 was omitted with little change in the results; therefore, the data reported here is with no H_2O_2 added. No H_2O_2 was added in the experiments with the breast cancer lines, since it has been reported that cancer cells express PS in vitro [13,14].

A typical equilibrium binding result is shown in Fig. 2 for the binding of the FP to endothelial cells. The non-specific binding, obtained in the absence of Ca^{2+}, is subtracted from the total binding to obtain the specific binding. The dissociation constant (K_d) for each cell line tested was obtained from the specific binding data using GraphPad Prism 5 software to give the following results: 0.5 ± 0.2 nM for endothelial cells (with saturation $A_{450\,nm} = 1.3$), 6.2 ± 1.6 nM for MCF-7 breast cancer cells (with saturation $A_{450\,nm} = 0.9$), and 4.9 ± 0.9 nM (with saturation $A_{450\,nm} = 1.8$) for MDA-MB-231 breast cancer cells. These results indicate that the binding of the FP to these cells is relatively strong. The binding of annexin V to endothelial cells was determined using the same procedures as for the FP. The K_d for the specific binding data was found using GraphPad Prism 5 software to be 0.9 ± 0.2 nM (with saturation $A_{450\,nm} = 1.2$), which is sim-
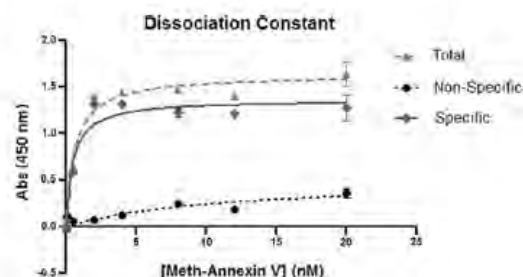
Fig. 2. Determination of FP binding strength to exposed PS on human endothelial cells. FP was biotinylated and streptavidin-HRP was used to quantify the binding. Total binding was obtained using 2 mM Ca^{2+} in the binding buffer. Non-specific binding was obtained by removing the Ca^{2+} from the binding buffer and replacing it with 5 mM of EDTA to chelate Ca^{2+}. Specific binding was obtained by subtracting the non-specific binding from the total binding. GraphPad Prism 5 software determined the specific binding to have a $K_d = 0.5 \pm 0.2$ nM. Data are presented as mean ± SE ($n = 3$).

ilar to the K_d determined for the binding of the FP to endothelial cells. Literature values of annexin V binding alone to endothelial cells have been reported from 2.7–15.5 nM [15,16].

### 3.3. Fusion protein binding stability

The FP bound per cell was studied over 3 days for the three cell lines to determine the stability of binding by measuring the absorbance at 450 nm, determined by the binding assay, and dividing by the fluorescence at 590 nm, determined by the Alamar Blue assay (Fig. 3). The data in Fig. 3 indicate the binding of FP declined over 3 days for all three cell lines, with the MDA-MB-231 cancer cells showing the most rapid decline; however, the FP was still present at day 3 for all three cell lines. Cell viability, as measured by the Alamar Blue assay, was found to be linearly proportional to the number of cells (data not shown).
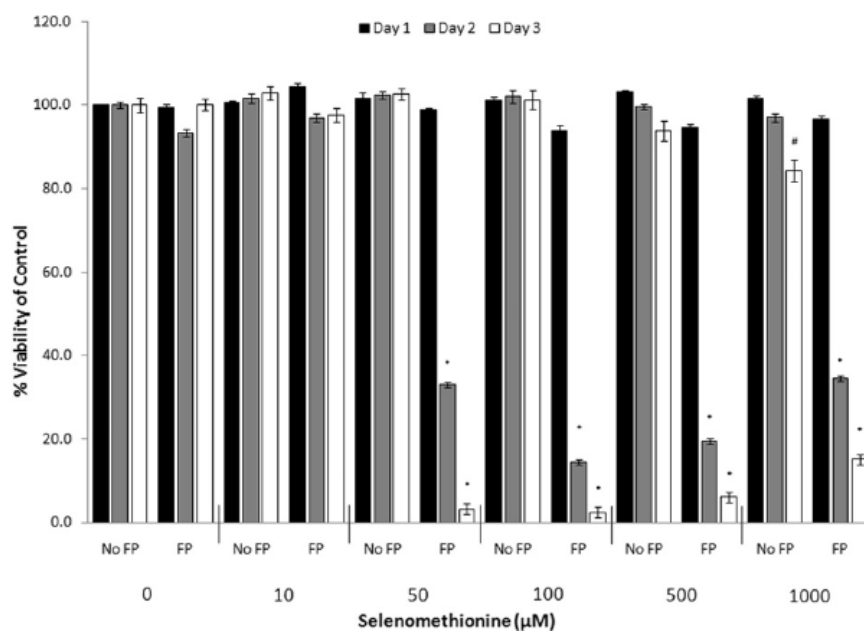
3.4. In vitro cytotoxicity of enzyme prodrug

The ability of the enzyme prodrug system to eliminate human endothelial cells and breast cancer cells was evaluated using a saturating concentration of fusion protein, followed by concentrations of SeMet ranging from 0 to 1000 μM (Figs. 4–6). The methionine concentration in the medium was set at a level (1000 μM) that would not lead to a significant decrease in cell viability because of methionine depletion with FP present.
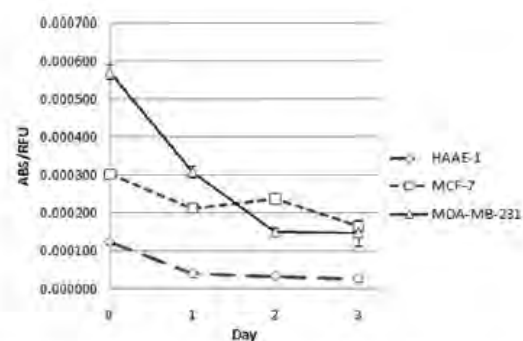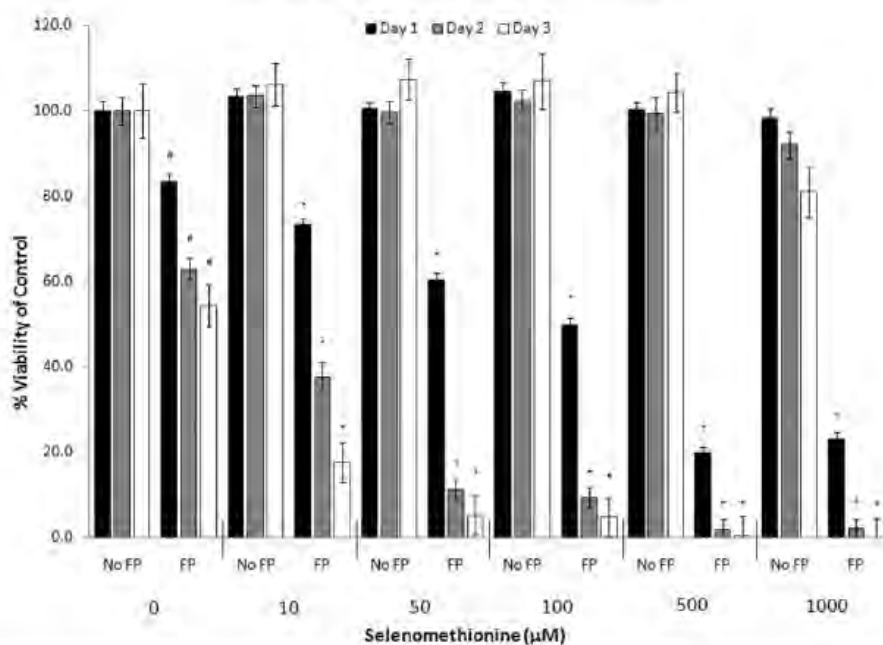

Fig. 3. Fusion protein binding stability. The Alamar Blue assay for cell viability was performed each day, followed by the binding assay to determine the duration of binding of annexin V to the exposed PS on the surface of each cell line. ABS/RFU is the absorbance at 450 nm, determined by the binding assay, divided by the relative fluorescence units at 590 nm, determined by the Alamar Blue assay. Data are presented as mean ± SE ($n = 3$).
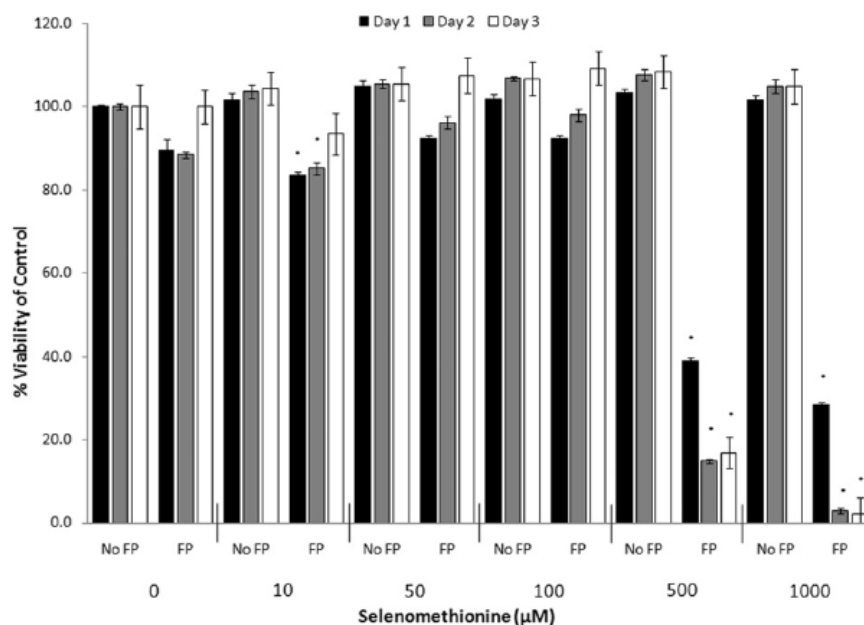
Fig. 4. Effect of SeMet conversion to methylselenol on HAAE-1 endothelial cells. Cells were grown in medium adjusted to 1000 μM of ʟ-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μM SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean ± SE ($n = 3$).

Fig. 5. Effect of SeMet conversion to methylselenol on MCF-7 breast cancer cells. Cells were grown in medium adjusted to 1000 μM of ʟ-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μM SeMet) on the same day, and statistical significance was denoted by # ($p < 0.001$). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean ± SE ($n = 3$).

B.D. Van Rite et al./Cancer Letters 301 (2011) 177–184

Fig. 6. Effect of SeMet conversion to methylselenol on MDA-MB-231 breast cancer cells. Cells were grown in medium adjusted to 1000 μM of L-methionine. Cell viability was assessed using the Alamar Blue assay for cell viability and normalized to the control (i.e. no FP and no SeMet). A one-way ANOVA was performed for statistical analysis. Cells treated with different SeMet concentrations but with no FP were compared to the control (no FP and 0 μM SeMet) on the same day, and statistical significance was denoted by # (p < 0.001). Cells treated with the FP were compared to cells with no FP on the same day at the same SeMet concentration, and statistical significance was denoted by * (p < 0.001). Data are presented as mean ± SE (n = 3).

Each of the cell lines metabolized the Alamar Blue to produce a fluorescence that was measured to quantify total cell viability. The fluorescence data from the Alamar Blue assay was expressed as a percentage of the fluorescence for the cells with no FP and 0 μM SeMet (control). Cells that were treated with different SeMet concentrations but no FP were compared to the control on the same day, whereas cells that had the FP were compared to cells with the same SeMet concentration but no FP on the same day.

The cytotoxicity results for the endothelial cells are shown in Fig. 4. Treatment with the FP gave significant cell killing for 500 and 1000 μM SeMet at days 1–3 (p < 0.001). With no FP present, significant cell cytotoxicity was not observed at the levels of SeMet tested.

Cytotoxicity results for the two breast cancer cell lines are shown in Figs. 5 and 6. For MCF-7 cells with FP present, there was significant killing at days 2 and 3 with 50–1000 μM SeMet (Fig. 5, p < 0.001). Cell killing without FP present was not significant on day 3 until the SeMet concentration reached 1000 μM. MDA-MB-231 cells showed a greater sensitivity to the SeMet than MCF-7 and endothelial cells (Fig. 6); significant cell cytotoxicity was observed with the FP present on days 1–3 with 10–1000 μM SeMet (p < 0.001). Even without the addition of the SeMet, binding of the FP alone produced significant cell killing. With no FP present, cell killing did not occur until the SeMet level was 1000 μM and was relatively small and not statistically significant (p < 0.001).

4. Discussion

In this study, we have shown that a novel FP developed for use in a new enzyme prodrug system binds relatively strongly to the surface of endothelial cells and two breast cancer cell lines. The K_d values for the two breast cancer cell lines are similar, while the K_d for the endothelial cells is about an order of magnitude lower, indicating much stronger binding. This low K_d value for the endothelial cells is favorable for this enzyme prodrug system that is directed to the tumor vasculature.

The fact that the FP exhibited the same degree of binding to endothelial cells with or without hydrogen peroxide leads us to believe that endothelial cells grown in vitro by our techniques expose PS on their surface. It has been shown previously in tumor-bearing mice that PS is exposed on the external surface of vascular endothelial cells in tumor blood vessels but not on endothelial cells outside of the tumor; this was demonstrated using biotinylated annexin V [3,4]. We have found that the strength and specificity of binding of annexin V by itself (i.e. not as part of a fusion protein) to endothelial cells is very similar to that of the FP using the same procedures; and, therefore, there is no reason to believe that the FP will bind with any different strength or specificity in vivo compared to annexin V.

This binding stability data (Fig. 3) indicate that the FP was still bound to the cells after 3 days. The effectiveness of the enzyme in the FP during the test period is reinforced by the cytotoxicity data for the endothelial cells and two breast cancer cell lines (Figs. 4–6) for the SeMet concentrations at which there was a statistically significant decline in cell viability compared to the same SeMet concentrations with no FP present on the same day (indicated by a *); for this data there was in almost every case a decline in cell viability from days 1 to 2 and from days 2 to 3. The medium for the cells was changed at days 1 and 2, which means that the enzyme would have to produce additional methylselenol for more cells to be killed; if additional methylselenol were not produced, then no

methylselenol would be present (since the medium was changed), and additional cell killing would likely not occur.

In the enzyme prodrug test of cytotoxicity for the endothelial cells, which are the primary target in the tumor for this enzyme prodrug therapy, the cytotoxic effect was evident after only one day of treatment at 500 μM SeMet. At either 500 μM or 1000 μM SeMet, there was no effect on endothelial cell viability with no FP present; this indicates that endothelial cells in the normal vasculature, which will not bind to the FP (since PS is not externalized), will not be affected by these concentrations of SeMet. At SeMet concentrations well below 500 μM, however, the cancer cells will be killed by toxic methylselenol being carried across the artery wall by fluid permeation; the effect of methylselenol on normal cells outside of the tumor is expected to be minimal or none because it will be greatly diluted by the bloodstream before it reaches the normal cells. MDA-MB-231 breast cancer cells were found to be the more sensitive to the enzyme prodrug treatment than MCF-7 breast cancer cells, even producing killing when the FP was bound but no SeMet prodrug was added.

We have not found evidence that the sensitivity of the three cell lines to the enzyme prodrug treatment is dependent on the degree of exposure of PS on the cell surface. The saturation $A_{450\,nm}$ values measured in the FP binding assay, which are a measure of the amount of FP bound to PS, do not correlate with the sensitivity to the enzyme prodrug treatment indicated in Figs. 4–6. Therefore, other cell properties besides the degree of PS exposure are contributing to the sensitivity to the effect of methylselenol.

Based on previous research, it is not expected that this enzyme prodrug therapy on the tumor vasculature will lead to toxicity to normal cells or organs. In a previous study using a monoclonal antibody to target anionic phospholipids in mice with implanted tumors, the growth of MDA-MB-231 and MDA-MB-435 breast tumors was inhibited, with no toxicity to the mice [17]. The blood vessels in MDA-MB-435 tumors implanted in mice have been shown to expose 35% of the anionic phospholipids [18]. PS is present at >10^6 molecules per cell [3], so even at 35% exposure, the number of anionic phospholipids on the surface of the cell is still very large.

Insight of how this enzyme prodrug system might be used therapeutically can be gained from the results of a previous study of treating mice with a methioninase cancer gene therapy with selenomethionine prodrug [7]. In this study, mice with implanted hepatoma tumor cells were treated with a recombinant adenovirus containing the L-methioninase gene and then were injected with SeMet once a day for 11 consecutive days, which led to inhibition of tumor growth and a significant prolongation of survival compared to mice not treated with SeMet. This dosing regimen is consistent with a previous finding that the selenium in human plasma peaks at 11 h on average after injection of sodium selenite [19]. No data have been reported on the half-life of methylselenol, probably because it is so highly reactive and therefore difficult to quantify and detect [20].

Several advantages of this new enzyme prodrug are envisioned. First, the FP can be easily administered through i.v. injection, and it will bind rapidly to its PS receptor in contact with the bloodstream. Second, there would be minimal side effects since the drug is produced locally in the tumor and the prodrug concentration would be at a level that would not affect cells in other tissues besides the tumor. Third, it allows rapid targeting of metastatic tumors anywhere in the body. Fourth, it combines enzyme prodrug therapy with methionine-depletion therapy. Finally, the killing of endothelial cells in the tumor vasculature will cause the release of tumor antigen directly into the bloodstream, potentially leading to a response by the immune system against cancer cells throughout the body, which can be boosted by the administration of immunoadjuvants [10]. One disadvantage, common to all enzyme prodrug systems, is that the enzyme is not a human protein and thus may cause an immune response. This can be overcome by conjugating the L-methioninase part of the FP to polyethylene glycol (PEG). L-methioninase has, in fact, been conjugated to PEG, and the administration of PEG-L-methioninase to monkeys has been shown to eliminate anaphylactic reactions [21].

In conclusion, based on the data presented there is evidence that this therapy will be able to cause cytotoxicity to tumors wherever they occur in the body with minimal side effects. This new enzyme prodrug therapy system to treat cancer appears promising.

Conflicts of interest

None declared.

Acknowledgement

References

[1] K.D. Bagshawe, Antibody-directed enzyme prodrug therapy (ADEPT) for cancer, Expert Rev. Anticancer Ther. 6 (2006) 1421–1431.
[2] G. Xu, H.L. McLeod, Strategies for enzyme/prodrug cancer therapy, Clin. Cancer Res. 7 (2001) 3314–3324.
[3] S. Ran, A. Downes, P.E. Thorpe, Increased exposure of anionic phospholipids on the surface of tumor blood vessels, Cancer Res. 62 (2002) 6132–6140.
[4] S. Ran, P.E. Thorpe, Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy, Int. J. Radiat. Oncol. Biol. Phys. 54 (2002) 1479–1484.
[5] N. Esaki, K. Soda, L-methionine gamma-lyase from Pseudomonas putida and Aeromonas, Methods Enzymol. 143 (1987) 459–465.
[6] N. Esaki, H. Tanaka, S. Uemura, T. Suzuki, K. Soda, Catalytic action of L-methionine gamma-lyase on selenomethionine and selenols, Biochemistry 18 (1979) 407–410.
[7] K. Miki, M. Xu, A. Gupta, Y. Ba, Y. Tan, W. Al-Refaie, M. Bouvet, M. Makuuchi, A.R. Moossa, R.M. Hoffman, Methioninase cancer gene therapy with selenomethionine as suicide prodrug substrate, Cancer Res. 61 (2001) 6805–6810.
[8] N. Yamamoto, A. Gupta, M. Xu, K. Miki, Y. Tsujimoto, H. Tsuchiya, K. Tomita, A.R. Moossa, R.M. Hoffman, Methioninase gene therapy with selenomethionine induces apoptosis in bcl-2-overproducing lung cancer cells, Cancer Gene Ther. 10 (2003) 445–450.
[9] A. Kim, J.H. Oh, J.M. Park, A.S. Chung, Methylselenol generated from selenomethionine by methioninase downregulates integrin expression and induces caspase-mediated apoptosis of B16F10 melanoma cells, J. Cell Physiol. 212 (2007) 386–400.
[10] W.R. Chen, R.L. Adams, R. Carubelli, R.E. Nordquist, Laser-photosensitizer assisted immunotherapy: a novel modality for cancer treatment, Cancer Lett. 115 (1997) 25–30.

[11] X.P. Zang, N.R. Palwai, M.R. Lerner, D.J. Brackett, J.T. Pento, R.G. Harrison, Targeting a methioninase-containing fusion protein to breast cancer urokinase receptors inhibits growth and migration, Anticancer Res. 26 (2006) 1745–1751.

[12] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[13] T. Utsugi, A.J. Schroit, J. Connor, C.D. Bucana, I.J. Fidler, Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes, Cancer Res. 51 (1991) 3062–3066.

[14] M. Sugimura, R. Donato, V.V. Kakkar, M.F. Scully, Annexin V as a probe of the contribution of anionic phospholipids to the procoagulant activity of tumour cell surfaces, Blood Coagul. Fibrin. 5 (1994) 365–373.

[15] W.L. van Heerde, S. Poort, Binding of recombinant annexin V to endothelial cells: effect of annexin V binding on endothelial-cell-mediated thrombin formation, Biochem. J. 302 (1994) 305–312.

[16] W.L. van Heerde, K.S. Sakariassen, Annexin V inhibits the procoagulant activity of matrices of TNF-stimulated endothelium under blood flow conditions, Arterioscler. Thromb. 14 (1994) 824–830.

[17] S. Ran, J. He, X. Huang, M. Soares, D. Scothorn, P.E. Thorpe, Antitumor effects of a monoclonal antibody that binds anionic phospholipids on the surface of tumor blood vessels in mice, Clin. Cancer Res. 11 (2005) 1551–1562.

[18] X. Huang, M. Bennett, P.E. Thorpe, A monoclonal antibody that binds anionic phospholipids on tumor blood vessels enhances the antitumor effect of docetaxel on human breast tumors in mice, Cancer Res. 65 (2005) 4408–4416.

[19] B.H. Patterson, O.A. Levander, K. Helzlsouer, P.A. McAdams, S.A. Lewis, P.R. Taylor, C. Veillon, L.A. Zech, Human selenite metabolism: a kinetic model, Am. J. Physiol. Regul. Integr. Comp. Physiol. 257 (1989) 556–567.

[20] K.T. Suzuki, K. Kuraksaki, S. Ogawa, N. Suzuki, Metaboloic transformation of methylseleninic acid through key selenium intermediate selenide, Toxicol. Appl. Pharmacol. 215 (2006) 189–197.

[21] Z. Yang, J. Wang, Q. Lu, J. Xu, Y. Kobayahsi, T. Takakura, A. Takimoto, T. Yoshioka, C. Lian, C. Chen, D. Zhang, Y. Zhang, S. Li, X. Sun, Y. Tan, S. Yagi, E.P. Frenkel, R.M. Hoffman, PEGylation confers greatly extended half-life and attenuated immunogenicity to recombinant methioninase in primates, Cancer Res. 64 (2004) 6673–6678.

APPENDIX II

Brent D. Van Rite and Roger G. Harrison, Annexin V-targeted Enzyme Prodrug Therapy Using Cytosine Deaminase in Combination with 5-Fluorocytosine, 2011, Cancer Letters, 307, 53-61.

Annexin V-targeted enzyme prodrug therapy using cytosine deaminase in combination with 5-fluorocytosine

Brent D. Van Rite, Roger G. Harrison *

Bioengineering Center and the School of Chemical, Biological and Materials Engineering, University of Oklahoma, Norman, OK, USA

ARTICLE INFO

ABSTRACT

A fusion protein, consisting of cytosine deaminase (CD) linked to human annexin V, was created for use in an enzyme prodrug therapy targeted to the tumor vasculature and associated cancer cells in the primary tumor and distant metastases. The major finding of this study is that the CD-annexin V fusion protein in combination with the prodrug 5-fluorocytosine has significant cytotoxic activity against endothelial cells and two breast cancer cells lines *in vitro* that expose phosphatidylserine on their surface. The cytotoxicity experiments verified this novel enzyme prodrug system has the ability to produce therapeutic levels of 5-fluorouracil and thus appears promising.

© 2011 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

The anti-metabolite 5-fluorouracil (5-FU) is widely used in the treatment of solid tumors, including those of the breast, gastrointestinal system, head and neck, and ovary [1]. It has been established that 5-FU is converted inside the cell to the active metabolites fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP) [2]. These metabolites misincorporate into RNA and DNA and inhibit the nucleotide synthesis enzyme thymidylate synthase. Unlike using a drug that rapidly disrupts the membrane, 5-FU requires DNA and RNA replication to induce toxicity.

Cytotoxicity to non-cancerous tissues limits the dosage and frequency of drug administration. Common side effects of 5-FU are bone marrow suppression leading to neutropenia and infections, and gastrointestinal toxicities such as stromatitis, nausea, vomiting, and diarrhea [3]. To circumvent problems related to systemic administration, new delivery strategies have been studied to produce 5-FU at the site of the tumor using an inert prodrug. As part of an enzyme prodrug system, 5-fluorocytosine (5-FC), a common antimicrobial compound known to exhibit high bioavailability [4], has been used in combination with cytosine deaminase (CD) to produce biologically relevant levels of 5-FU locally. CD has been used in antibody-directed enzyme prodrug therapy (ADEPT) by conjugating it to a single chain fragment variable (scFv) antibody [5,6]. In ADEPT, however, the antibody-enzyme complex must diffuse across the vascular wall to reach the tumor cells. In a study of the local distribution of a monoclonal antibody injected i.v. in patients with malignant melanoma, the antibody was found to be heterogeneously distributed through the tumor, which was not primarily due to heterogeneity of antigen expression but could have been a result of differences in capillary wall permeability [7].

Other studies have used CD-fusions for suicide gene-directed enzyme prodrug therapy (GDEPT) [8,9] and viral-directed enzyme prodrug therapy (VDEPT) [10,11]. Gene delivery is limited, however, by an inability to transfect large number of cells efficiently, while viral vectors have the potential of inducing severe immune responses and

---

* Corresponding author. Address: School of Chemical, Biological and Materials Engineering, University of Oklahoma, 100 E. Boyd, Room T335, Norman, OK 73019, USA. Tel.: +1 405 325 4367.

E-mail address: rharrison@ou.edu (R.G. Harrison).

also suffer from a limited amount and size of plasmid DNA [12].

As a potential new enzyme prodrug system designed to have cytotoxic effects on the primary breast tumor and distant metastases with minimal side effects, we linked yeast CD to human annexin V protein, known to bind specifically to the anionic phospholipid phosphatidylserine (PS), to create a fusion protein (FP). Yeast CD was chosen since previous work has demonstrated a greater ability of yeast CD to convert 5-FC to 5-FU than bacterial CD [13]. PS is exposed on the surface of vascular endothelial cells within the blood vessels of tumors, but not on normal endothelium [14,15]. There is also abundant evidence that PS is expressed on the outer surface of tumor cells [16,17]. Intravenous injection of the FP will allow annexin V to bind rapidly to exposed PS on endothelial cells in the tumor vasculature, and the FP will also be transported from the bloodstream to tumor cells via the well known enhanced permeability and retention (EPR) effect [18]. Following clearance of unbound FP, 5-FC would be injected and converted to 5-FU within the tumor both at the surface of the tumor vasculature and at the surface of the cancer cells.

In the present study, human endothelial cells and MCF-7 and MDA-MB-231 breast tumor cells were used to characterize binding and stability of binding of this novel yeast CD-annexin V fusion protein. FP binding data (K_D) suggests relatively strong binding to exposed PS, and the FP stays bound at least 3 days. The addition of 5-FC prodrug following FP binding to PS demonstrated the ability to kill the three cell lines by generating toxic 5-FU.

2. Materials and methods

2.1. Materials

Oligonucleotide primers were synthetically produced by Integrated DNA Technologies (Coralville, IA). Linear pET-30 Ek/LIC vector, T4 DNA polymerase, HRV 3C protease, and NovaBlue and BL21(DE3) *Escherichia coli* cells were obtained from EMD Chemicals (Gibbstown, NJ). BamHI restriction enzyme and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). HAAE-1 endothelial cells were from Coriell Cell Repositories (Camden, NJ). MCF-7 and MDA-MB-231 cells and cell culture media were obtained from the American Type Culture Collection (Manassas, VA). Streptavidin-HRP was purchased from KPL (Gaithersburg, MD). PCR and plasmid purification kits were from Qiagen (Vista, CA). Alamar Blue solution was obtained from BioSource (Camarillo, CA). 5-FC and 5FU were obtained from Sigma-Alrdrich (St. Louis, MO).

2.2. Construction of recombinant expression plasmid

The expression vector pET-30 Ek/LIC/yCD-ANX, encoding the cytosine deaminase-annexin V fusion protein (FP), was constructed in the following manner: The DNA sequences encoding for the FP were amplified from pQE30Xa/yCD [19] (obtained from Dr. Maurizio Cianfriglia at the Superior Health Institute, Rome, Italy) and pET-22b(+)/STFANX (obtained from Dr. Stuart Lind at the

University of Colorado) by the polymerase chain reaction using the Expand High Fidelity PCR system (Boehringer Mannheim, Indianapolis, IN). The following PCR primers were used to create the fusion protein gene, connected by a flexible, six-amino acid linker: (a) 5′ primer for cytosine deaminase: 5′-*GAC/GAC/GAC/AAG/ATG*/**CTT/GAA/GTC/CTC/TTT/CAG/GGA/CCC**/GTG/ACA/GGG/GGA/ATG/GCA/AGC-3′ (b) 3′ primer for cytosine deaminase: 5′-GC/CGC/ATT/GGA/TCC/AGA/ACC/GTC/GCC/CTC/ACC/AAT/ATC/TTC/AAA/CC-3′ (c) 5′ primer for annexin V: 5′-CG/ATT/CGC/GGA/TCC/GCA/CAG/GTT/CTC/AGA/GGC-3′ (d) 3′ primer for annexin V: 5′-*GA/GGA/GAA/GCC/CGG*/TTA/GTC/ATC/TTC/TCC/ACA/GAG/C-3′. The cytosine deaminase primers incorporated a 5′ LIC cloning site (italics), an HRV 3C protease site (bold), and a 3′ BamHI site (underlined). The annexin V primers added a 5′ BamHI site (underlined) and a 3′ LIC cloning site (italics).

Using the QIAquick PCR purification kit, PCR products were purified, digested with BamHI restriction enzyme, and purified with the same kit. Pure, digested genes were ligated using T4 DNA ligase and run on an agarose gel. The proper fused gene segment was cut from the gel and purified using the QIAquick gel purification kit. After annealing the pure cytosine deaminase-annexin V fusion gene to the pET-30 Ek/LIC linear vector using T4 DNA polymerase to create sticky ends, competent NovaBlue cells were transformed. Following successful transformation, plasmids containing the proper fusion gene insert were extracted from the NovaBlue cells using the QIAprep plasmid purification protocol. For host protein expression, *E. coli* BL21(DE3) cells were transformed with the plasmid. The final vector contains integrated thrombin and enterokinase cleavage sites, an N-terminal His-tag sequence for easy purification, and an engineered HRV 3C protease cleavage site for cleaving the sequence LEVLFQ↓GP at the start of the cytosine deaminase-annexin V gene. Sequencing by the Oklahoma Medical Research Foundation (Oklahoma City, OK) verified the gene sequence to be correct.

2.3. Expression and purification of recombinant protein

Recombinant cytosine deaminase-annexin V FP was produced and purified using the procedure of Zang et al. [20], using immobilized metal affinity chromatography (IMAC) with immobilized Ni^{2+} to isolate the FP. After cleavage by HRV 3C protease, purified FP is collected in the flow-through of a second IMAC. Uncleaved FP and the protease, which retain the His-tag, bind to the IMAC column and allow for collection of only cleaved FP.

2.4. Protein content and enzymatic activity determination

The BCA Protein assay from Thermo Scientific (Waltham, MA) was used for all protein determinations throughout the purification using bovine serum albumin (BSA) as the standard. Samples were analyzed by denaturing gel electrophoresis using the SDS–PAGE method with Coomassie blue staining [21]. The cytosine deaminase enzyme activity was measured as described by Senter et al. [22]. Enzyme solution (225 μl) was added to PBS at 37 °C containing 0.5 mg/ml 5-FC for a total volume of 1 ml and

incubated at 37 °C for 30 min. A 50 µl sample was quenched in 1 ml of 0.1 N HCl. From that, 250 µl was removed, and its absorbance was measured at 255 and 290 nm using a BioTek Synergy HT microtiter plate reader (Winooski, VT). The concentration of 5-FU in quenched solutions was calculated from the formula given by Senter et al. One unit of enzyme activity is defined as 1 µmol of 5-FU formed/min at 37 °C.

2.5. Cell culture

Human HAAE-1 aortic endothelial cells were grown in F-12 K medium with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS), 0.03 mg/ml endothelial cell growth supplement, and 0.1 mg/ml heparin. MCF-7 human breast cancer cells were maintained as monolayer cultures in Eagle's minimum essential medium containing Earle's balanced salt solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and supplemented with 10% FBS and 0.01 mg/ml bovine insulin. MDA-MB-231 human breast cancer cells were grown in Leibovitz's L-15 medium supplemented with 10% FBS and 2 mM L-glutamine. Penicillin (100 U/ml) and streptomycin (100 µg/ml) were also added to each medium. HAAE-1 and MCF-7 cells were grown at 37 °C in a 5% CO_2 atmosphere, while MDA-MB-231 cells were grown without additional CO_2 at 37 °C, as recommended by the American Type Culture Collection.

2.6. Fusion protein binding assays

Each cell line was grown in T-75 flasks to 70–80% confluence, transferred to 24-well culture plates at 5×10^4 cells/well, and grown to 80–85% or 100% (control) confluence. Cells were fixed to the plate using 0.25% glutaraldehyde in binding buffer (PBS with 2 mM Ca^{2+}), and unreacted aldehyde groups were quenched using binding buffer with 50 mM NH_4Cl. Prior to adding the FP, a 1 h pretreatment of BSA (0.5%) in PBS was carried out at 37 °C. Varying concentrations (0–20 nM) of biotinylated FP, using SureLINK Chromophoric Biotin (KPL) for biotinylation, were diluted in binding buffer containing 0.5% BSA and incubated at 37 °C for 2 h. After washing with binding buffer with 0.5% BSA, streptavidin-HRP was added at 2 µg/ml and incubated at room temperature for 1 h. Following a final wash with binding buffer, HRP was quantified by adding the chromogenic substrate O-phenylenediamine (0.4 mg/ml) in 0.05 mM phosphate–citrate buffer (pH 5.0) containing 0.012% hydrogen peroxide to initiate the reaction. After 30 min at room temperature in the dark, 100 µl of the solution was transferred to a transparent 96-well plate, and the absorbance was read at 450 nm on a microtiter plate reader. To determine non-specific binding, the same procedure was performed with no Ca^{2+} and 5 mM EDTA in the binding buffer. All experiments had a blank that was subjected to the same procedure but with no FP added.

As further confirmation of FP binding to the cell membrane, MDA-MB-231 and endothelial cells were incubated for 2 h with FP tagged with FITC, following the protocol provided with the FITC labeling kit from Pierce (Rockford, IL). A fluorescence microscope equipped with a Nikon DXM1200F digital camera was used for documentation of fluorescence.

2.7. Binding stability assay

A modified binding assay was used to determine the duration of FP binding to the surface of the three cell lines. Cells on 24-well plates were first incubated for 2 h at 37 °C in a saturating concentration of biotinylated FP (100 nM) in complete growth medium with 2 mM Ca^{2+}. The Alamar Blue assay was done on separate sets of cells at day 0, 1, 2 and 3 to determine viability, followed by fixing with 0.25% glutaraldehyde in binding buffer. Unreacted aldehyde groups were quenched by incubation with 50 mM NH_4Cl in binding buffer. The binding of FP was then quantified using streptavidin-HRP and OPD as above. The Alamar Blue assay was performed by adding Alamar Blue solution to each well to give 10% Alamar Blue and then incubated for 4 h at 37 °C. A portion of the solution (250 µl) was transferred to an opaque 96-well plate, and the fluorescence was read at 590 nm using excitation at 530 nm. The blank consisted of wells containing only medium and Alamar Blue solution.

2.8. In vitro enzyme prodrug cytotoxicity to cells

The experiment was carried out over 9 days using the same set of cells. Cells were grown and plated out in 24-well plates with respective growth media using the same procedure as for the FP binding assay above. Each medium was supplemented with 2 mM Ca^{2+} since annexin V is Ca^{2+} dependent. On day 0, the cells were incubated in medium containing 100 nM FP for 2 h at 37 °C. The plates were washed and medium containing 5-FC or 5-FU varying from 0 to 2000 µM was added. Cells receiving 5-FU did not receive FP. Each day throughout the experiment, the medium was replaced with fresh medium containing 5-FC or 5-FU. The Alamar Blue assay was performed on all wells on days 3, 6, and 9 (as above). After the fluorescence readings on days 3 and 6, the plates were washed, and the cells were incubated with new FP using the same procedures as before. The cells were washed again, and fresh medium containing appropriate levels of 5-FC or 5-FU was added before further incubation.

2.9. Data analysis

All assays included wells in triplicate. To test differences in cell viability, a one-way ANOVA employing a Tukey–Kramer multiple comparisons test was performed using GraphPad InStat software (GraphPad; La Jolla, CA) with a significance level of $p < 0.001$.
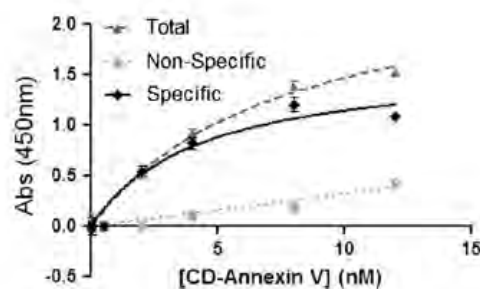
3. Results

3.1. Protein expression and purification

A representative SDS–PAGE gel of the FP purification is shown in Fig. 1. Following expression of the fusion protein, the BL21 (DE3) cells were harvested by centrifugation, resuspended in lysis buffer and
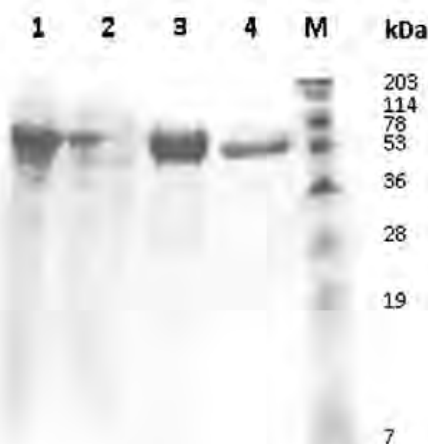
Fig. 1. SDS–PAGE gel of cytosine deaminase-annexin V purification. An 8% gel with Coomassie blue staining was used to determine approximate protein molecular masses in 10 μl samples. *Lane 1*, soluble proteins; *lane 2*, first chromatography flow-through; *lane 3*, first chromatography elution; *lane 4*, second chromatography flow-through; *M* marker proteins with molecular masses indicated in kiloDaltons (5 μl).

Fig. 2. Determination of FP binding strength to exposed PS on MDA-MB-231 breast cancer cells. FP was biotinylated, and streptavidin–HRP was used to quantify the binding. Total binding was obtained using 2 mM Ca^{2+} in the binding buffer. Non-specific binding was obtained by removing the Ca^{2+} from the binding buffer and replacing it with 5 mM of EDTA to chelate Ca^{2+}. Specific binding was obtained by subtracting the non-specific binding from the total binding. GraphPad Prism 5 software determined the specific binding to have a $K_d = 4.2 \pm 1.8$ nM (SE). Data are presented as mean ± SE ($n = 3$).

### 3.2. Specific binding and dissociation constant determination

The ability of the FP to bind to exposed PS on endothelial cells and breast cancer cells *in vitro* was determined by equilibrium binding experiments using increasing concentrations of biotinylated FP. We previously determined that endothelial cells cultured *in vitro* with our methods expose PS [23]; thus hydrogen peroxide was not used to induce exposure of PS. It has been reported that cancer cells express PS *in vitro* [16,17].

A sample equilibrium binding result is shown in Fig. 2 for MDA-MB-231 breast cancer cells. The non-specific binding, obtained in the absence of Ca^{2+} and presence of EDTA, is subtracted from the total binding to obtain the specific binding. The dissociation constant (K_D) for each cell line tested was obtained from the specific binding data by GraphPad Prism 5 software to give the following results: 1.5 ± 0.2 nM (with saturation $A_{450nm} = 0.6$) for endothelial cells, 0.6 ± 0.4 nM (with saturation $A_{450nm} = 0.5$) for MCF-7 breast cancer cells, and 4.2 ± 1.8 nM (with saturation $A_{450nm} = 1.2$) for MDA-MB-231 breast cancer cells, indicating relatively strong binding between the FP and PS. In a control test, the specific binding assay was performed on endothelial cells that were 100% confluent. The result was that the total and non-specific data were essentially identical, indicating no specific binding and therefore a lack of PS expression on the cell surface (data not shown).

To illustrate binding of the FP, MDA-MB-231 cells were incubated with FITC-labeled FP. Fig. 3 shows a light microscopy image (left) of MDA-MB-231 cells and a fluorescence microscopy image (right) of FP bound to PS on the cell surface. Endothelial cells incubated with

sonicated. The resulting soluble protein fraction is shown in lane 1. The His-tag attached to the fusion protein was bound to the Ni^{+2} column during the first chromatography step to allow some of the unwanted proteins to flow through into waste (lane 2), and then it was eluted (lane 3). After cleavage with the protease, the FP eluted during the flow-through (lane 4) of a second chromatography, while other proteins remained in the column. Dialysis of the flow-through, sterile filtration, and lyophilization ended the purification process. The yield of purified FP was 50 mg from 1 L of culture medium. The purity of the FP (lane 4) was estimated to be >96% using UN-SCAN-IT densitometry software analysis (Silk Scientific, Inc). The theoretical molecular weight of the FP monomer is 53 kDa, which is consistent with the SDS–PAGE result for the purified FP (lane 4). The lyophilized FP was determined to have a cytosine deaminase specific activity of 18 U/mg of protein. The overall recovery yield of cytosine deaminase activity was found to be >90%. Enzyme activity remained relatively constant when stored for 4 months in lyophilized form at −80 °C.

Fig. 3. Visualization of fluorescent FP binding to MDA-MB-231 breast cancer cells. Cells were seeded onto a cover glass and allowed to adhere. FP labeled with FITC was added, allowed to bind for 2 h, and washed to remove unbound FP. Corresponding light (left) and fluorescence (right) images were acquired with a 40× objective to verify FP binding to the cell surface. The bar represents 100 μm.
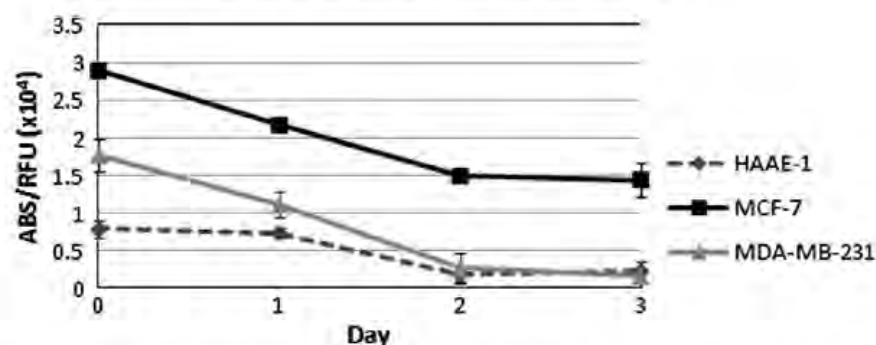
Fig. 4. Fusion protein binding stability. The Alamar Blue assay for cell viability was performed each day, followed by the binding assay to determine the duration of binding of the FP to exposed PS on the surface of each cell line. ABS/RFU is the absorbance at 450 nm, determined by the binding assay, divided by the relative fluorescence units at 590 nm, determined by the Alamar Blue assay. Data are presented as mean ± SE ($n = 3$).

FITC-labeled FP were also observed to be fluorescent only at the cell surface when they were 70% confluent (data not shown). No fluorescence was observed when endothelial cells were 100% confluent, indicating no binding of the FP and thus no expression of PS on the surface of the cells.

3.3. Fusion protein binding stability

The FP bound per cell was studied over 3 days for the three cell lines to determine the stability of binding. The binding assay data (absorbance at 450 nm) was divided by the Alamar Blue fluorescence data for cell viability (relative fluorescence at 590 nm) (Fig. 4). The endothelial cells and

MCF-7 cancer cells had the lowest and highest amount of initial binding, respectively. For all three cell lines, the binding of the FP per cell declined over 3 days; however, binding was still measureable after 3 days. The number of viable cells and fluorescence signal produced by the Alamar Blue assay were found to exhibit a linear relationship (data not shown).

3.4. In vitro cytotoxicity of enzyme prodrug

Cell viability was determined on days 3, 6, and 9 to demonstrate the cytotoxic effects of 5-FC, with or without the fusion protein, against human endothelial cells and breast cancer cells (Figs. 5–7). In addition,
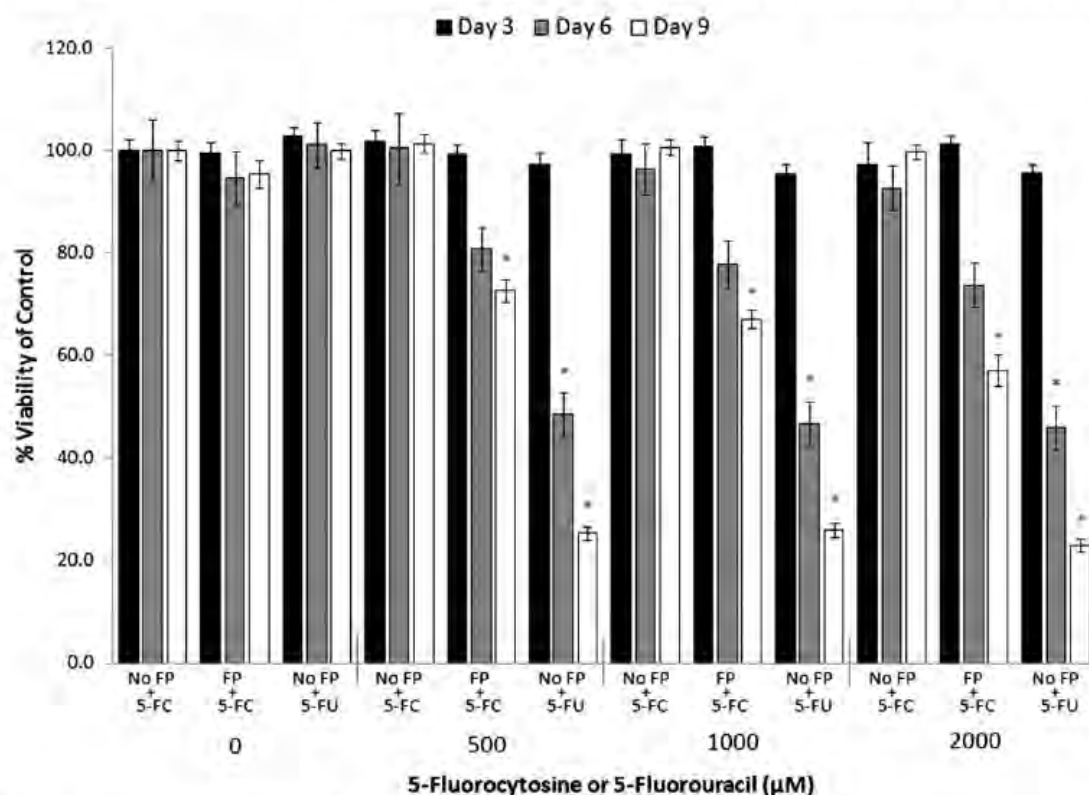
Fig. 5. Effect of 5-FC conversion to 5-FU on HAAE-1 endothelial cells. Cell viability was assessed using the Alamar Blue assay and normalized to the control (i.e. no FP and no 5-FC). A one-way ANOVA was performed for statistical analysis. Cells treated with the FP or cells treated with only 5-FU were compared to cells with no FP on the same day at the same 5-FC concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean ± SE ($n = 3$).
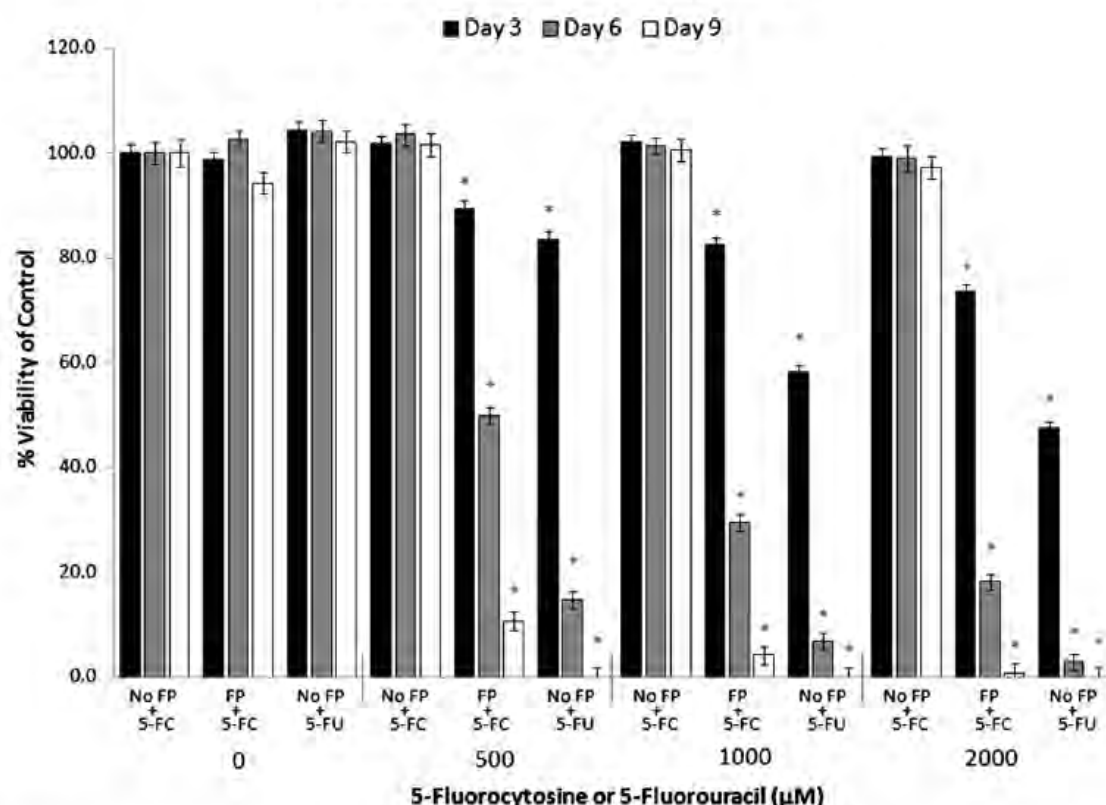
Fig. 6. Effect of 5-FC conversion to 5-FU on MCF-7 breast cancer cells. Cell viability was assessed using the Alamar Blue assay and normalized to the control (i.e. no FP and no 5-FC). A one-way ANOVA was performed for statistical analysis. Cells treated with the FP or cells treated with only 5-FU were compared to cells with no FP on the same day at the same 5-FC concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean ± SE ($n = 3$).

treatment with commercially available 5-FU was performed. The viability data is presented as the percentage of fluorescence produced relative to control samples (no FP and 0 µM 5-FC). All of the experimental groups were compared to the control for that particular day (no FP and the same 5-FC concentration). For all three cell lines, no significant cell death was found with 5-FC present and FP absent, or with 5-FC absent but FP present.

For the endothelial cells, there was negligible cell killing in the first 3 days of the experiments; however, significant killing ($p < 0.001$) was observed on day 9 for cells with FP plus 5-FC and on days 6 and 9 for cells with 5-FU. Killing was observed to be dose-dependent. At the same 5-FC concentration and incubation time, producing 5-FU using the FP and 5-FC gave less cytotoxicity than with 5-FU directly.

The two breast cancer cell lines exhibited similar patterns of cell death. Both MCF-7 and MDA-MB-231 cells receiving the FP plus 5-FC showed significant cell killing that was dose-dependent, compared to the control group, on days 3, 6, and 9 (Figs. 6 and 7, $p < 0.001$). By day 9, treatment with the FP plus 5-FC resulted in less than 10% viability at 500 µM 5-FC for MCF-7 cells and at 1000 µM 5-FC for MDA-MB-231 cells. As for the endothelial cells, the combination of 5-FC and the FP gave less cytotoxicity than with 5-FU directly at the same 5-FC concentration and incubation time.

4. Discussion

The major finding to emerge from this study is that CD–annexin V fusion protein in combination with the prodrug 5-FC has significant cytotoxic activity against endothelial cells and two breast cancer cells lines that expose PS on their surface. This means that this system has the potential for a dual effect of cutting off the tumor's blood supply and acting directly on cancer cells as a result of the EPR effect that will allow the FP to be transported from the bloodstream to the tumor, while minimizing damage to normal organs and tissue.

The yield and purity of the FP are very good (50 mg per liter of culture broth and >96% purity, respectively), and its specific activity of 18 U/mg is consistent on a molar basis with that previously reported for purified yeast CD of 56 U/mg [24]. The theoretical molecular masses of the FP and CD are 106 kDa and 34, respectively. Therefore, the specific activity we would expect for the FP based on the previously reported specific activity of CD and the two molecular masses is 18 U/mg, or exactly what we measured.

Based on the size of the FP, the diameter of this molecule is approximately 7 nm, assuming a sphere with a density of 1 g cm^{-3} [25]. Blood vessel gaps and fenestrations in solid tumors are known to be leaky and range from 100 to 600 nm [26]. Based on this data, it is expected that in addition to the FP binding to surface of the vascular endothelial cells in the tumor, the FP will pass though the vascular wall and bind to the surface of tumor cells directly. When administered intravenously, 5-FC will be able to reach the FP, whether it is in the primary tumor or a distant
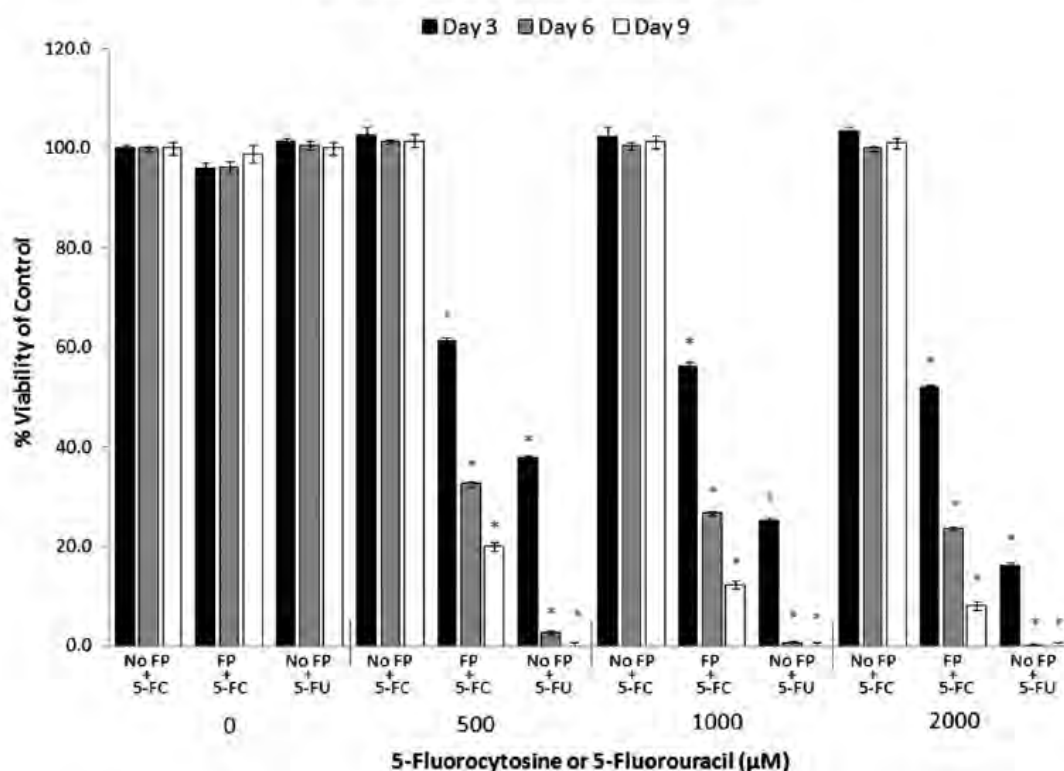
Fig. 7. Effect of 5-FC conversion to 5-FU on MDA-MB-231 breast cancer cells. Cell viability was assessed using the Alamar Blue assay and normalized to the control (i.e. no FP and no 5-FC). A one-way ANOVA was performed for statistical analysis. Cells treated with the FP or cells treated with only 5-FU were compared to cells with no FP on the same day at the same 5-FC concentration, and statistical significance was denoted by * ($p < 0.001$). Data are presented as mean $\pm$ SE ($n = 3$).

metastasis. Formation of 5-FU at the endothelial or cancer cell surface will result in uptake of 5-FU and the cascade of events ending in inhibition of RNA and DNA processes and cell death.

Critical for the success of this enzyme prodrug system, the binding strength of FP to externally positioned PS was determined to be in the low nanomolar range for endothelial cells, indicating high affinity for PS. Literature values of annexin V alone binding to endothelial cells have been reported from 2.7 to 15.5 nM [27,28]. The K_D values for the three cell lines studied were either below or the low end of this range of previously reported values, which may be the result of the FP existing as a dimer. The K_D values are also comparable to that of a different fusion protein we produced containing annexin V at the carboxy-terminus [23]. Additionally, we produced annexin V alone and found that it had a similar K_D [23].

Through the use of fluorescence microscopy, we observed that the FP was bound to the surface of breast cancer cells with PS exposed and not internalized (Fig. 3). By remaining on the cell surface and not being internalized, where it would be digested, the FP is able to convert 5-FC to 5-FU and thus lead to cytotoxicity of the cell and other cells surrounding it, by a bystander effect. Previous research has shown that annexin V by itself (i.e. not fused

to another protein) is taken up by endocytic vesicles after a two-dimensional crystallization on the cell surface [29]. Based on the images in Fig. 3, we can conclude that annexin V being part of a fusion protein has prevented it from forming two-dimensional crystals and then being internalized.

This study was able to mimic the extent of PS exposure previously found in vivo in both the normal vasculature and tumor vasculature [14,15]. When the endothelial cells were 100% confluent, no binding of the FP was observed in both the equilibrium binding assay and the microscopic fluorescence assay, indicating no PS exposure as is characteristic of normal vasculature; while for endothelial cells that were dividing and non-confluent, a strong binding of the FP was observed in the equilibrium binding assay, and binding was visualized at the cell surface using fluorescent FP.

When the endothelial cells were 100% confluent, no binding of the FP was observed in both the equilibrium binding assay and the microscopic fluorescence assay. Thus, our tests with 100% confluent endothelial cells mimic the normal vasculature, where it has been found that PS is not exposed on the endothelial cell surface in vivo [14,15].

Comparison between the efficacy of the FP/5-FC treatment that generates 5-FU and treatment with 5-FU by

itself at the same concentration showed that there was higher toxicity with 5-FU alone (Figs. 5–7). This finding is consistent with other reports comparing a drug's toxicity with the toxicity of the drug generated from its prodrug [13,30].

It is interesting to compare our results with another study in which two cancer cell lines were targeted using a fusion protein consisting of a single chain anti-CEA antibody linked to the amino-terminus of yeast CD [6]. Mel 5P human melanoma and LoVo human colon adenocarcinoma cells were incubated with this fusion protein, washed, and then incubated for 4 days with 5-FC at various concentrations. At the highest 5-FC concentration of 780 μM, the survival of both cell lines was approximately 70%. By contrast, in our study for incubation for 3 or 6 days and 5-FC concentration of 500 or 1000 μM, the survival for MCF-7 and MDA-MB-231 cells was in the range of 29–89% and 26–61%, respectively. From our study, it is evident that it is necessary to allow sufficient time for the enzyme prodrug therapy in order to achieve complete or near complete cell killing, which is likely because this therapy is dependent on targeting cells that are dividing. After 9 days of treatment with the CD-annexin V/5-FC enzyme prodrug therapy, the degree of cell death was very high, with 4% and 12% cell viability at 1000 μM 5-FC for MCF-7 and MDA-MB-213 cancer cells, respectively, and with no significant toxicity without the FP at this same 5-FC concentration.

We have recently tested another novel enzyme prodrug system that consisted of ʟ-methioninase–annexin V/selenomethionine, where the selenomethionine is converted to toxic methylselenol by the enzyme [23]. Similar cytoxicity results were obtained using the same three cell lines. The presently discussed system is more of a known type of treatment, since 5-FU has been used clinically much more than methylselenol, while the ʟ-methioninase–annexin V/selenomethionine system has the advantage that it also degrades ʟ-methionine, which leads to death of methionine-dependent cancer cells.

Previous analyses have been done to determine the half-life of 5-FC and 5-FU. Patients given 5-FC orally with normal kidney function reportedly had a 5-FC half-life of 3–4 h, while those with poor renal function extended the half-life out to several days [4,31]. 5-FU has a very short biological half-life, found to be only 5–10 min [32]. Even with short life in plasma, studies have shown accumulation of 5-FU in tumor tissues, while no accumulation in normal tissues (not rapidly proliferating tissue) of rabbits was observed [33]. Since 5-FU is generated *within* the tumor using the cytosine deaminase-annexin V/5-FC system, side effects such as bone marrow suppression are expected to be low or nonexistent. Also, having a prodrug with a relatively long half-life will allow for the frequency of prodrug administration to be reduced, which can mean fewer hospital visits for patients undergoing treatment with this enzyme prodrug system.

Following administration of the FP, an immune response may occur, as is normal for any foreign molecule. PEGylation of CD has been done previously with little change to the enzyme properties [34]. An FP consisting of PEGylated CD linked to human annexin V would not be expected to produce an immune response; *in vivo* tests would be required to confirm this.

In summary, we have constructed a fusion protein consisting of a protein used to target exposed PS and CD enzyme that converts prodrug 5-FC to its toxic metabolite 5-FU. Recombinant production resulted in a highly active enzyme with functional annexin V, demonstrated by its ability to bind to PS exposed by endothelial cells and breast cancer cells. Cytotoxicity experiments verified this novel enzyme prodrug system has the ability to produce therapeutic levels of 5-FU and thus appears promising as a means of eliminating breast tumors wherever they occur in the body with minimal side effects.

Acknowledgements

References

[1] M.D. Wyatt, D.M. Wilson 3rd, Participation of DNA repair in the response to 5-fluorouracil, Cell Mol. Life Sci. 66 (2009) 788–799.

[2] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-fluorouracil: mechanisms of action and clinical strategies, Nat. Rev. Cancer 3 (2003) 330–338.

[3] W.Y. Cheung, R.A. Fralick, S. Cheng, The confused cancer patient: a case of 5-fluorouracil-induced encephalopathy, Curr. Oncol. 15 (2008) 234–236.

[4] R. Cutler, A. Blair, M. Kelly, Flucytosine kinetics in subjects with normal and impaired renal function, Clin. Pharmacol. Ther. 24 (1978) 333–342.

[5] V. Coelho, J. Dernedde, U. Petrausch, H. Panjideh, H. Fuchs, C. Menzel, S. Dubel, U. Keilholz, E. Thiel, P. Deckert, Design, construction, and in vitro analysis of A33scFv::CDy, a recombinant fusion protein for antibody-directed enzyme prodrug therapy in colon cancer, Int. J. Oncol. 31 (2007) 951–957.

[6] S. Zamboni, A. Mallano, M. Flego, A. Ascione, M.L. Dupuis, M. Gellini, S. Barca, M. Cianfriglia, Genetic construction, expression, and characterization of a single chain anti-CEA antibody fused to cytosine deaminase from yeast, Int. J. Oncol. 32 (2008) 1245–1251.

[7] S. Del Vecchio, J.C. Reynolds, J.A. Carrasquillo, R.G. Blasberg, R.D. Neumann, M.T. Lotze, G.J. Bryant, R.J. Farkas, S.M. Larson, Local distribution and concentration of intravenously injected 131I-9.2.27 monoclonal antibody in human malignant melanoma, Cancer Res. 49 (1989) 2783–2789.

[8] H. Zeng, Q. Wei, R. Huang, N. Chen, Q. Dong, Y. Yang, Q. Zhou, Recombinant adenovirus mediated prostate-specific enzyme prodrug gene therapy regulated by prostate-specific membrane antigen (PSMA) enhancer/promoter, J. Androl. (2007) 827–835.

[9] M. Chaszczewska-Markowska, K. Stebelska, A. Sikorski, J. Madej, A. Opolski, M. Ugorski, Liposomal formulation of 5-fluorocytosine in suicide gene therapy with cytosine deaminase–for colorectal cancer, Cancer Lett. 262 (2008) 164–172.

[10] B. Huber, C. Richards, E. Austin, Virus-directed enzyme/prodrug therapy (VDEPT). Selectively engineering drug sensitivity into tumors, Ann. NY Acad. Sci. 716 (1994) 104–114.

[11] J. Harris, A. Gutierrez, H. Hurst, K. Sikora, N. Lemoine, Gene therapy for cancer using tumour-specific prodrug activation, Gene Ther. 1 (1994) 170–175.

[12] Y. Singh, M. Palombo, P.J. Sinko, Recent trends in targeted anticancer prodrug and conjugate design, Curr. Med. Chem. 15 (2008) 1802–1826.

[13] E. Kievit, E. Bershad, E. Ng, P. Sethna, I. Dev, T. Lawrence, A. Rehemtulla, Superiority of yeast over bacterial cytosine deaminase for enzyme/prodrug gene therapy in colon cancer xenografts, Cancer Res. 59 (1999) 1417–1421.

[14] S. Ran, A. Downes, P.E. Thorpe, Increased exposure of anionic phospholipids on the surface of tumor blood vessels, Cancer Res. 62 (2002) 6132–6140.

[15] S. Ran, P.E. Thorpe, Phosphatidylserine is a marker of tumor vasculature and a potential target for cancer imaging and therapy, Int. J. Radiat. Oncol. Biol. Phys. 54 (2002) 1479–1484.

[16] T. Utsugi, A.J. Schroit, J. Connor, C.D. Bucana, I.J. Fidler, Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes, Cancer Res. 51 (1991) 3062–3066.

[17] M. Sugimura, R. Donato, V.V. Kakkar, M.F. Scully, Annexin V as a probe of the contribution of anionic phospholipids to the procoagulant activity of tumour cell surfaces, Blood Coagul. Fibrin. 5 (1994) 365–373.

[18] H. Maeda, G.Y. Bharate, J. Daruwalla, Polymeric drugs for efficient tumor-targeted drug delivery based on EPR-effect, Eur. J. Pharm. Biopharm. 71 (2009) 409–419.

[19] A. Mallano, S. Zamboni, G. Carpinelli, F. Santoro, M. Flego, A. Ascione, M. Gellini, M. Tombesi, F. Podo, M. Cianfriglia, Generation and characterization of a human single-chain fragment variable (scFv) antibody against cytosine deaminase from yeast, BMC Biotechnol. 8 (2008) 68.

[20] X.P. Zang, N.R. Palwai, M.R. Lerner, D.J. Brackett, J.T. Pento, R.G. Harrison, Targeting a methioninase-containing fusion protein to breast cancer urokinase receptors inhibits growth and migration, Anticancer Res. 26 (2006) 1745–1751.

[21] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[22] P.D. Senter, P.C. Su, T. Katsuragi, T. Sakai, W.L. Cosand, I. Hellstrom, K.E. Hellstrom, Generation of 5-fluorouracil from 5-fluorocytosine by monoclonal antibody-cytosine deaminase conjugates, Bioconjug. Chem. 2 (1991) 447–451.

[23] B. Van Rite, Y. Lazrak, M. Pagnon, N. Palwai, L. Neves, P. McFetridge, R. Harrison, Enzyme prodrug therapy designed to target L-methioninase to the tumor vasculature, Cancer Lett. 301 (2011) 177–184.

[24] M. Hayden, P. Linsley, A. Wallace, H. Marquardt, D. Kerr, Cloning, overexpression, and purification of cytosine deaminase from saccharomyces cerevisiae, Protein Expres. Purif. 12 (1998) 173–184.

[25] R. Fournier (Ed.), Basic Transport Phenomena in Biomedical Engineering, Taylor & Francis Group, New York, 2007.

[26] A. Lila, T. Ishida, H. Kiwada, Targeting anticancer drugs to tumor vasculature using cationic liposomes, Pharm. Res. (2010) 1171–1183.

[27] W.L. van Heerde, K.S. Sakariassen, H.C. Hemker, J.J. Sixma, C.P. Reutelingsperger, P.G. de Groot, Annexin V inhibits the procoagulant activity of matrices of TNF-stimulated endothelium under blood flow conditions, Arterioscler. Thromb. 14 (1994) 824–830.

[28] W.L. van Heerde, S. Poort, C. van't Veer, C.P. Reutelingsperger, P.G. de Groot, Binding of recombinant annexin V to endothelial cells: effect of annexin V binding on endothelial-cell-mediated thrombin formation, Biochem. J. 302 (Pt 1) (1994) 305–312.

[29] H. Kenis, H. van Genderen, A. Bennaghmouch, H.A. Rinia, P. Frederik, J. Narula, L. Hofstra, C.P. Reutelingsperger, Cell surface-expressed phosphatidylserine and annexin A5 open a novel portal of cell entry, J. Biol. Chem. 279 (2004) 52623–52629.

[30] P. Erbs, E. Regulier, J. Kintz, P. Leroy, Y. Poitevin, F. Exinger, R. Jund, M. Mehtali, In vivo cancer gene therapy by adenovirus-mediated transfer of a bifunctional yeast cytosine deaminase/uracil phosphoribosyltransferase fusion gene, Cancer Res. 60 (2000) 3813–3822.

[31] J. Schönebeck, A. Polak, M. Fernex, H. Scholer, Pharmacokinetic studies on the oral antimycotic agent 5-fluorocytosine in individuals with normal and impaired kidney function, Chemotherapy 18 (1973) 321–336.

[32] S. Li, A. Wang, W. Jiang, Z. Guan, Pharmacokinetic characteristics and anticancer effects of 5-Fluorouracil loaded nanoparticles, BMC Cancer 8 (2008) 103–111.

[33] W. Wolf, C.A. Presant, K.L. Servis, A. el-Tahtawy, M.J. Albright, P.B. Barker, R. Ring 3rd, D. Atkinson, R. Ong, M. King, et al., Tumor trapping of 5-fluorouracil: in vivo 19F NMR spectroscopic pharmacokinetics in tumor-bearing humans and rabbits, Proc. Natl. Acad. Sci. USA 87 (1990) 492–496.

[34] M. Xiong, G. Kwon, PEGylation of yeast cytosine deaminase for pretargeting, J. Pharm. Sci. 94 (2005) 1249–1258.